ROLE OF SHEAR STRESS IN THE DIFFERENTIAL REGULATION OF ENDOTHELIAL CATHEPSINS AND CYSTATIN C

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Manu Omar Platt

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ROLE OF SHEAR STRESS IN THE DIFFERENTIAL REGULATION OF ENDOTHELIAL CATHEPSINS AND CYSTATIN C

Approved by:

Dr. Hanjoong Jo, Advisor
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Zorina S. Galis
Eli Lilly
Indiana University

Dr. Robert M. Nerem
School of Mechanical Engineering
School of Chemical Engineering
Georgia Institute of Technology

Dr. W. Robert Taylor
School of Medicine
Division of Cardiology
Emory University

Dr. Guo-Ping Shi
Cardiovascular Medicine
Brigham and Women's Hospital
Harvard Medical School

Date Approved: June 26, 2006

To James for the discipline,
Sandy for the dedication,
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LIST OF SYMBOLS AND ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ApoE	Apolipoprotein E
AV	aortic valve
AVF	Arteriovenous fistula
BMP-4	Bone Morphogenic Protein-4
Cath	cathepsin
CM	carboxymethylated
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC	Endothelial Cell
ECGS	Endothelial cell growth supplement
ECM	Extracellular Matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular-Signal Regulated Kinase-1/2
FBS	Fetal Bovine Serum
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycans
HRP	horse radish peroxidase
ICAM-1	Intercellular adhesion molecule – 1

IEL	Internal elastic lamina
IFN-γ	Interferon gamma
IHC	immunohistochemistry
IL-1β	Interleukin – 1 beta
JNK	c-jun N-Terminal Kinase
LDL	Low-Density Lipoprotein
LDLR	Low density lipoprotein receptor
LS	Unidirectional laminar shear stress
MAEC	Murine aortic endothelial cells
MAP Kinase	Mitogen activated protein kinase
MEK	MAP kinase kinase
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
NF-κB	Nuclear factor-κB
OS	oscillatory shear stress
OxLDL	oxidized low-density lipoprotein
PBS	Phosphate buffered saline
PI3K	Phosphatidyl inositol-3 kinase
PVDF	polyvinyldifluoride
r	cone radius
RGD	arginine-glycine-aspartic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
siRNA	small interfering ribonucleic acid
SMC	Smooth muscle cell
St	static
τ	shear stress
TEHV	tissue engineered heart valve
TGF-β	Transforming growth factor β
TIMP	Tissue Inhibitor of Metalloproteinases
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
VCAM-1	Vascular cell adhesion molecule – 1
WCL	whole cell lysate
Z-DEVD	Benzyloxycarbonyl-Asp-Asn-Val-Asp
Z-FR-AMC	Benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin
Z-RR-AMC	Benzyloxycarbonyl-Arg-Arg-7-amino-4-methylcoumarin
α	Cone angle
μ	viscosity
v	Kinematic viscosity
ω	angular velocity

SUMMARY

Endothelial cells are exposed to oscillatory shear stress at sites prone to atherosclerotic and aneurysmal formation. These cells sense low or oscillatory shear stress and signal the blood vessel wall to remodel to maintain an open lumen for the passage of blood. Remodeling involves protease degradation and reconstruction of the extracellular matrix structural components. Several different proteases are capable of degrading these elastin and collagen components including matrix metalloproteases and lysosomal cysteine proteases (commonly called cathepsins). Although they were first identified in the lysosomes, these proteins are secreted by certain cells and are potent elastases and collagenases. To regulate their extracellular proteolytic activity, cystatin C, their most powerful inhibitor, exists in relatively high concentrations to reversibly bind and inhibit them. Once cystatin C is downregulated, however, the cathepsins can now degrade and remodel the structural components of the blood vessel wall to advance atherosclerotic plaque development. This downregulation of cystatin C by an endothelial cell mediated pathway is a key point in plaque development and must be addressed and understood instead of solely implicating matrix metalloproteases in this disease process; new therapeutic targets can then be designed.

The novel and significant finding of this study is that exposure to atheroprotective unidirectional laminar shear stress (LS) decreases elastase and gelatinase activities of endothelial cells through a shear stress mediated reduction in cathepsin activity. Oscillatory shear stress increases this activity. Cathepsins K, L, and S, elastinolytic and collagenolytic proteases, are shown to be regulated by shear stress in these studies at the level of the protein and activity. Cathepsin B was not responsive to shear stress. The

endogenous inhibitor of the cathepsin family, cystatin C, was found to be inversely regulated by shear stress; LS increased its secretion by endothelial cells while OS decreased it. Binding of free cystatin C in the conditioned media to carboxymethylated papain coated agarose beads led to an increase in cathepsin activity since the available cathepsin was not inhibited. Additional roles for cathepsins K, L, and S were found in endothelial cell alignment in response to unidirectional laminar shear stress, endothelial cell migration, and programmed cell death.

Immunolabeling of cathepsins K, L, and S in mouse aortic endothelial cells after exposure to OS, LS, or St conditions verify that cathepsins L and S are predominantly lysosomal in location intracellularly even though they are secreted by the cells as well. Cathepsin K staining suggests that it is not only intracellularly, but also bound on the cell membrane as detected in plasma membrane fractions of the endothelial cells and visualized with subcellular proteome extraction followed by immunostaining.

To verify these findings in human samples, immunohistochemical analysis of cystatin C and cathepsin K was performed on human coronary arteries. Cathepsin K stained strongly in the endothelial layer of vessels with degraded internal elastic lamina while cystatin C staining intensity was strongest overlying minimally diseased vessels. This again corroborates the inverse regulation of cathepsin K and cystatin C in endothelial cells of large arteries and identifies the cathepsin family of proteases as potential targets for therapeutic intervention of atherosclerotic plaque development at sites of disturbed flow.

CHAPTER 1

INTRODUCTION

Cardiovascular disease and shear stress studies have been linked for the last two decades. Endothelial cells line the blood vessel wall and serve several functions; they inhibit platelet aggregation and coagulation, selectively transport macromolecules from the blood into the blood vessel wall, and most importantly for this study, sense hemodynamic flow. It has been well established that differences in local hemodynamics induce different cell signaling pathways in endothelial cells (1-6). A unidirectional laminar shear stress signals the endothelial cells to produce nitric oxide, a vasodilator, and other atheroprotective agents (7). Oscillatory shear stress induces apoptotic signals and cell adhesion molecule expression to encourage monocyte adhesion leading to an inflammatory response and atherosclerosis. One such protein induced by oscillatory shear stress is BMP-4 which upregulates ICAM-1 leading to monocyte adhesion (8). This review will focus on the shear stress mediated cardiovascular pathologies such as atherosclerosis, abdominal aortic aneurysms (AAA), and valvular diseases and the proteases responsible for the pathological remodeling that occurs during their development.

Shear stress

Shear stress can be defined as a tangential force over a surface area, and in the vasculature, this corresponds to the dragging force of blood as it flows over the blood vessel wall, the valve leaflets, or the cardiac wall. The viscosity of the blood and the velocity gradient are proportional to the shear stress at the wall:

$$\tau = \mu \, du/dr$$

and can be described by

$$\tau_{wall} = \frac{32\mu Q}{\pi D^3}$$

for steady laminar flow in a straight tube (9).

In vitro determination of flow profiles in different cardiovascular geometries and anatomical locations have mostly been carried out in the lab with their recreation in glass tube models. Particle tracking velocimetry, hydrogen bubbles, and other methods (as reviewed by Ku (9) provide a visual picture of the three-dimensional flow while laser Doppler anemometry, pulsed Doppler ultrasound, and Hot-film anemometry were used to determine the velocity profiles (10-13).

Clinical determination of wall shear stress used calculations from velocity measurements detected using Doppler ultrasound and magnetic resonance imaging (MRI) (14, 15). These tools can be combined with computational fluid dynamics (16, 17) to mathematically predict the velocity and shear stress profiles at distinct locations and defined geometries. It is important to note calculations of wall shear stress by these methods still contain a degree of error.

Shear stress and cardiovascular diseases

The importance of shear stress in vascular biology and pathophysiology has been highlighted by the focal development patterns of atherosclerosis in hemodynamically defined regions. For example, the regions of branched and curved arteries exposed to disturbed flow conditions including oscillatory and low mean shear stresses (OS)

correspond to athero-prone areas. In contrast, straight arteries exposed to pulsatory, high levels of laminar shear stress (LS) are relatively well protected from atherosclerotic plaque development (18).

Several human and animal studies have also demonstrated that atherosclerotic lesions and abdominal aortic aneurysms (AAA) occur in regions exposed to unstable flow conditions including flow reversal, low mean wall shear stress and high oscillatory shear index (16, 19-23). Diminished flow has also been shown to cause AAA in patients with above the knee amputations making them five times more prone to this disease (24), and patients with peripheral vascular disease also have reduced flow and increased risk of developing AAA (25). In contrast, relatively high levels of laminar shear stress were shown to reduce AAA progression in rat experimental models (26).

Aortic valves and mitral valves function in complex hemodynamic environments due to the opening and closing of the valves. Different sides of the valve leaflets each experience a different flow environment (27), and this alters the endothelial behavior on the valve leaflets (28). In valves, the fibrosa layer which is on the aortic side of the aortic valve is exposed to disturbed flow and prone to development of calcification and valvular atherosclerosis, believed to develop due to the hemodynamics to which they are exposed (28). Their preferential development on the aortic side suggests that hemodynamic contribution is important in their valvular formation as well.

The common factor in each of these cardiovascular diseases is that endothelial cells line these structures and are the fluid shear stress mechanosensor. Endothelial cell response to shear stress has been studied from the transcriptional level all the way up to proliferation and migration. Overwhelming evidence indicates that high levels of

unidirectional laminar shear stress increases nitric oxide production causing vasodilation and arterial health (1, 6). Pro-inflammatory cytokines, such as BMP-4 (8, 29), and cell adhesion molecules, such as VCAM-1, are turned down under LS to retard the initial inflammatory responses that lead to atherosclerotic plaque formation. More importantly for this study, high levels of unidirectional laminar shear stress inhibit the vascular remodeling that is found in regions exposed to disturbed or stagnant flow. At sites of disturbed flow, intimal thickening and breakdown of the elastic laminae structures are two of the main remodeling processes that involve proteases responding to changes in shear stress.

Atherosclerosis

Atherosclerotic plaque formation requires major remodeling of the blood vessel wall. Monocytes enter the blood vessel wall using the cell adhesion molecules expressed by the endothelium under oscillatory shear stress and in response to oxidized LDLs. They differentiate into macrophages and begin to phagocytose lipids in the wall, transform into foam cells, and eventually rupture and release their contents back into the subintimal space (30). Cytokines such as IL-1β and IFN-γ released from the macrophages and foam cells reach the smooth muscle cells in the medial layer of the blood vessel wall and signal them to change phenotype. Usually, the smooth muscle cells in this layer are of a contractile phenotype, serving to maintain tension in the artery (31), but they change to a migrating, secretory phenotype. As the macrophages secrete superoxides and proteolytic enzymes, they create an acidic milieu to activate proteolytic enzymes (32), which can degrade the elastic layer separating the smooth muscle cells

from the endothelial cells. Those smooth muscle cells that have converted to the secretory phenotype are now capable of migrating through the fragmented elastic laminar layer into the intimal region of the blood vessel wall where the atherosclerotic plaque is forming. Positive feedback continues as they also rupture once they have internalized too many lipids releasing all of their contents as well into the advancing plaque region.

Abdominal aortic aneurysms

Aneurysms are defined as a permanent dilatation of a blood vessel, and one that occurs in the infrarenal aorta is termed an abdominal aortic aneurysm. Elastin and collagen destruction in this section of the aorta are characteristic of AAA. Fragmentation of the elastic fibers in the medial layer is an initial step in AAA development, but loss of the structural support of types I and III collagen in the adventitia as they are degraded eventually causes rupture of the aneurysm, from which 65% of patients die (33).

The abdominal aorta is subject to disturbed flows because of anatomical factors such as the diaphragm, the lumbar curvature, and low resistance in the renal arteries. These cause the formation of strong vortices, skewed flow, and reverse flow into the renals, respectively (9) all leading to oscillatory and low average wall shear stress. Glass tube models of the abdominal aorta were used to visualize these flow changes, and they have been verified in humans (34) and correlated with atherosclerosis in autopsy patients (21, 35).

Valve Disease

The aortic valve experiences a complex hemodynamic environment. Three distinct flow regions exist in the aortic valve: the aortic wall of the sinus of valsalva characterized with low, disturbed flow; the side of the leaflets facing the aorta with low shear, laminar flow and the side facing the ventricle with high shear, laminar flow (36). Myxomatous heart valve disease is characterized by collagen fragmentation and accumulation of proteoglycans and eventually leads to prolapse of the leaflets (37); it generally occurs focally in the mitral valve suggesting a role for hemodynamics in its development, and proteases have been identified as being upregulated in myxomatous heart valve leaflets (38). Senile valve disease occurs preferentially on the aortic side as well and is characterized by fibro-calcific formations, inflammation, and neovascularization in its later stages (37); all of these have been linked to disturbed flow in blood vessels.

Model Systems

In vitro shear stress systems.

The two most widely used devices to actuate shear stress on endothelial cells *in vitro* are the cone and plate viscometer and the parallel plate flow chamber. For the cone and plate apparatus, endothelial cells are cultured to confluence on a tissue culture dish prior to placing a cone into the medium and rotating it at the appropriate speed to obtain arterial levels of shear stress on the adhered endothelial cells with cone angle and media

viscosity considered in the design of the cone (39-41). Magnetic stir bars or programmable motors are used to control the speeds of rotation. Engineering analysis of these systems yielded equations that can be used to calculate the shear stress at the endothelial cell surface and the limits to which the cone and plate can actuate physiological, laminar shear stress profiles (2, 39, 41). This is the preferred method of exposing endothelial cells to shear stress in our lab and will be used for the studies in this manuscript. Cone and plate shear apparatus have been used in studies of cell adhesion molecules (42, 43) , secreted protein and activity (44, 45), and shear stress induced signaling cascades (46-49).

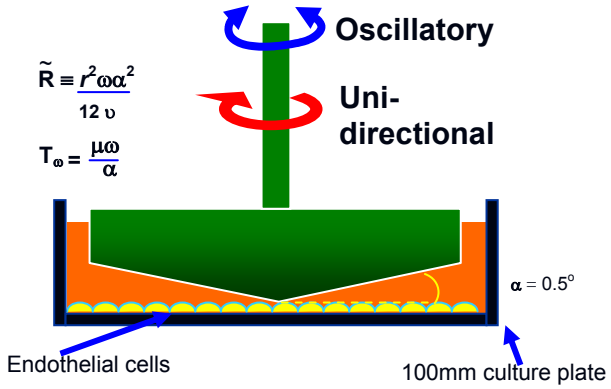

Figure 1.1 Schematic of cone and plate shear apparatus. Ř is a non-dimensional parameter, analogous to the Reynolds number, indicating laminar azimuthal flow for values of Ř < 1 (r=radius of cone, ω=angular velocity, α=cone angle, ν=kinematic viscosity). Within these parameters the plate shear stress. τ_w, is uniform across the plate and equal to μω/α (μ=viscosity, ω=angular velocity, α=cone angle) (2).

Parallel plate flow chambers are governed by a different set of equations and assumptions. Endothelial cells are cultured on glass slides and placed in a sterile chamber, and media is pumped across the cells to actuate the shear stress. Pressure difference and height above the glass slide determine the level of shear stress experienced by the cells (50). Cell proliferation, calcium flux, viscoelastic properties, intracellular

signaling, and other concepts have been studied with these flow chambers (51-58). They have also been used to study valve leaflet responses to shear stress (59).

Ex vivo models

Organ culture systems offer added advantages over cell systems because the interaction of the different cells that comprise the blood vessel or the valve leaflet can be observed while recapitulating the three-dimensional flow environment such as was done in the original glass casts used for hemodynamic investigations. Porcine (60, 61), mouse (62), and human (63) *ex vivo* systems have been described to culture entire arteries for extended periods of time. Gambillara et al have cultured left common porcine carotid segments in a perfusion system and even studied oscillatory shear effects (64, 65). Alternatively, groups are using co-culture models of smooth muscle cells with endothelial cells as a method to study cell interactions. Some of these models seek to recreate the three-dimensional architecture while others focus on the cytokines and crosstalk between the cells (66, 67). Heart valve *ex vivo* studies have been valuable model systems as well supplying information on matrix remodeling due to pressure and shear stress (68-70).

In vivo models

Flow cessation models

Ligation of the carotid artery is used *in vivo* to block flow and examine the responsive vascular remodeling. In mice, sufficient blood flow still reaches the brain through other vessels so that the ligation is not fatal. This model of flow cessation causes

induction of proteases, intimal hyperplasia, macrophage infiltration, all with an intact endothelium (71-74).

Low flow

Low flow models *in vivo* have been accomplished with partial ligations of carotid arteries (75) or by ligation of internal carotid artery and of the three of the other branches of the external carotid artery (76). Partial outflow occlusion models also lead to lower flow through the artery of interest (77). Intimal hyperplasia and protease induction are seen in these animal models as well. Humans with peripheral vascular disease experience low flow in the abdominal aorta, and it also subjects them to a greater risk of developing abdominal aneurysms (25).

High flow

Arteriovenous fistula (AVF) operations are prevalent animal surgeries performed to generate high flows *in vivo*. Usually, the fistula is created between the carotid artery and the jugular vein. This procedure is more often performed in rabbits (77, 78), but has also been created in Sprague Dawley rats (79) and mice leading to an increase of shear stress from 35 to 261 dynes/cm^2 in right common carotid artery AVF (80). Combination studies have been performed with partial ligation of the left carotid artery after ligations of the internal and left external carotids. This decreased flow in the left carotid artery by 90% but caused an increase in flow in the right carotid artery (75). Effects of both low and high flow in the same animal were then investigated.

Oscillatory flow

Besides the natural geometry of the vascular tree that induce oscillatory flow such as the carotid bifurcations, lesser curvature of the aorta, and the sharp turns of the coronary arteries, Shu Chien's group has developed a U shaped titanium clip that can be placed on the rat aorta to induce oscillatory flow and unidirectional flow *in* vivo at straight sections of the aorta (81).

Proteases and Remodeling

Matrix metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs)

MMPs are the class of zinc and calcium dependent endopeptidases that have been highly investigated in vascular biology and matrix remodeling. Currently, 14 MMPs have been studied in vascular cells. They are synthesized as zymogens, with a propeptide that must be cleaved to activate the latent enzyme. MMPs and members of other protease families are capable of this cleavage putting into motion a cascade of proteolytic activity to increase their combined activity. Together, as a family, MMPs are capable of degrading all of the components of the vascular extracellular matrix and are classified according to their substrate preference: collagenases, gelatinases, stromelysins, membrane-type MMPs, and others (82).

TIMPs are the endogenous, potent inhibitors of MMPs and are also studied in vascular physiology to observe any shift in the balance from inhibition to proteolysis by this family. There is a 1:1 stoichiometric binding of TIMP to MMP that involves the N-

terminal cysteine displacement of the water molecule at the active site from the zinc ion (83).

Endothelial cells, smooth muscle cells, and macrophages all secrete MMPs (84) in the vascular wall in response to different stimuli as reviewed by Newby including mechanical injury, mechanical forces such as shear stress and stretch; inflammatory cytokines interleukin-1 and tumor necrosis factor-α; and growth factors platelet-derived growth factor and fibroblast growth factor-2 (82). Many of these factors induce vascular remodeling which can be healthy or pathological (84). Atherosclerotic remodeling leads to breakdown of the elastin and collagen components of the vascular wall and change in SMC phenotype from contractile to secretory and migratory; MMP involvement has been shown with knockout mice backcrossed to ApoE knockout mice fed a high fat diet. MMP-9 and ApoE double knockout mice showed reduced plaque burden and atherosclerosis compared to ApoE knockout mice controls (85). The elastic lamina was also more intact with the genetic absence of MMP-9 or MMP-12 (85). Conversely, genetic null TIMP-1 mice had increased aneurysmal formation but reduced atherosclerotic plaques when crossed with ApoE knockout mice and fed a high fat diet (86) showing the importance of these matrix proteases in pathological vascular remodeling and how their inhibition contributes to vascular stability.

Hemodynamic regulation of MMPs has also been described using the above listed shear stress systems. *In vitro* studies of a murine endothelial cell line showed that laminar shear stress upregulated MMP-2 and MMP-9, oscillatory flow increased MMP-9 (87). Shear stress did not increase MMP-2 and MMP-9 activity in an *ex vivo* porcine artery culture, but transmural pressure did induce their activity (60). Flow cessation

models heavily implicate induction of MMP expression and activity in vascular smooth muscle cells as a mechanism for intimal hyperplasia (71-73, 88). Low flow induced intimal hyperplasia upregulates MMP-9 (75, 76), but high flow also increases MMP-2 and -9 activity and degradation of the internal elastic lamina in a rabbit model of AVF (89). With balloon injured right common carotid arteries in rabbits, MMP-2 activity increased by 186% left in normal flow; partial outflow occlusion after injury to maintain low flow increased its activity by 366% while high flow brought on by ligation of the left common carotid caused only a 39% increase (77).

MMPs are also involved in remodeling of heart valves under disease conditions. Myxomatous heart valves are floppy heart valves due to the degradation of elastin and collagen components and MMP-1, -2, -9, and -13 have been identified immunohistochemically (38), and MMP-1 and -2 have been identified in healthy human heart valve leaflets (90). Bicuspid aortic valves have impaired hemodynamics and lead to failure, remodeling, and disease; MMP-2 and -9 were increased in human bicuspid aortic valves compared to human triscuspid aortic valves (91).

Cathepsins and cystatins

The cathepsin family of cysteine proteases has been studied far less than MMPs in vascular remodeling and literature is particularly sparse in flow mediated cathepsin dependent vascular remodeling.

Cysteine proteases are involved in the catalysis of other proteins using their respective OH- and SH- groups at the active site to cleave amide bonds. The two main families of cysteine proteases are the caspases and the papain superfamily. Caspases are well known to be involved in apoptotic cell death pathways, and the papain superfamily

(comprising the cathepsins) are mainly found in lysosomal compartments, but have been shown to be secreted by macrophages and osteoclasts. "Cathepsin" is the main term used for this superfamily of cysteine proteases, and their activity is highly regulated. Cathepsins function best in an acidic pH, and they are only weakly active at a neutral pH. They prefer a reducing environment and are synthesized as an inactive precursor, requiring proteolytic cleavage of a propeptide region. In addition, there are several inhibitors constantly present in high ratios to the cathepsins (32, 92-95). Ultimately, expression of a cathepsin does not mean it will play a physiological role; it must be moved to an acidic environment, activated, and be free from inhibition. When the regulatory mechanisms fail, cathepsins are capable of causing pathological damage. Emphysema (92), Alzheimer's disease (96), pycnodystosis (97), tumor metastasis (92, 98) rheumatoid arthritis (99), muscular dystrophy (92), and now atherosclerosis, the subject of this study, show cysteine protease involvement (92).

Cathepsin S is a potent elastase and has the special characteristic that at neutral pH, it is still active, making it unique among the papain family of cysteine proteases (100). Shi et al. found it in atherosclerotic plaques (101). There was decreased elastic lamina breakdown and reduced plaque development in Cathepsin S and LDL receptor double knockout mice (102). There was also decreased microvessel growth without cathepsin S. Macrophages have been identified as one of the cell types that secrete this cysteine protease pericellularly (31). It has already been linked to emphysema (92), the destruction of the alveoli, and is now being investigated for its role in atherosclerosis.

Cathepsin L is as potent an elastase as cathepsin S, but it only functions in acidic pH (103). It is also secreted by macrophages at sites of inflammation (31). Although

secreted in the proform, it can be activated in the acidic, local environment created by the macrophages. Active cathepsin L in aortic aneurysms further implicates this cysteine protease in cardiovascular disease (104). Disease pathologies include tumor metastasis, bone resorption, as well as implications in arthritis (92).

Cathepsin B is one of the more abundant cathepsins with lysosomal concentrations as high as one millimolar (94). Much work has been done on the collagenolytic abilities of cathepsin B and its role in tumor metastasis by degrading the basement membrane of tumor cells (105). Once again, pathologies develop from cathepsins being placed outside of the lysosomes; cathepsin B can be relocated to the tumor cell surface and bound to annexin II where it is activated (106). Additionally in vivo atherosclerotic imaging has identified the presence of cathepsin B (107).

Cathepsin K is a potent (94, 108, 109) collagenase and elastase that we have found is secreted into the conditioned media by MAECs. It is the most potent collagenase, capable of cleaving mature type I collagen in the native triple helix and in the telopeptide regions (110) while other collagenases can only cleave at one or the other. Although this protease was first characterized regarding osteoclast bone resorption, it has also been located in atherosclerotic plaques (101). ApoE null mice that were also null for Cathepsin K had smaller plaques, increased collagen content, and less elastic lamina breaks (111).

Cathepsins B, K, L, and S are elastinolytic and collagenolytic, proving capable of degrading the main structural components of the blood vessel wall (32, 104, 107, 112). The acidic environment created by macrophage secretions in the plaque-forming, intimal region would provide an adequate environment for cathepsin activity. Atherosclerotic

lesions, now being classified as sites of inflammation, are shown to be acidic (113). Macrophages acidify the local environment through a vacuolar type ATPase pump that is also activated by close contact with elastin, creating acidic microenvironments optimal for cysteine protease function (114). Release of these proteases into the intimal region of the vessel wall by endothelial cells demands that an inhibitor be present to keep their activity at a minimum and to avoid a pathological condition. Cystatin C is just such an inhibitor.

Cystatins are the group of inhibitors of lysosomal cysteine proteases, of which, cystatin C is the most powerful (100). This 13 kD protein is translated with a 26 amino acid signal peptide that targets it for secretion approximately one hour after post-translational processing (116). At this point its three-dimensional conformation forms a wedge shape that will block the substrate binding cleft of the cathepsin in a tight, reversible fashion(117) with an inhibition constant on the subnanomolar range (118). In the context of atherosclerosis, its production and secretion has been studied in smooth muscle cells of blood vessel walls. TGF-β, a cytokine present in blood vessel walls, induces an increased secretion of cystatin C from smooth muscle cells although there is no detectable change in its mRNA levels (112). Cystatin C has also been found in normal blood vessel tissue sections in the medial layer, but was absent in atherosclerotic sections (101, 112). Its production by endothelial cells remains elusive. We have found that cystatin C is upregulated by endothelial cells in response to laminar shear stress through DNA microarray (Table 1.1).

Table 1.1. DNA microarray data from mouse aortic endothelial cells exposed to 24 hours of unidirectional laminar shear, oscillatory shear stress, or static, no flow conditions. Median values from 3 independent experiments are shown.

Description	Laminar Shear Fold Change	Oscillatory Shear Fold Change
CYSTATIN C	2.45	0.92
CATHEPSIN L	2.46	1.94
CATHEPSIN S	1.67	0.71
CATHEPSIN B	0.85	1.05
CATHEPSIN K	1.04	2.62

Overall hypothesis

Unidirectional laminar shear stress inhibits cathepsin levels and maintains high levels of cystatin C in the blood vessel wall through an endothelial cell mediated pathway, but oscillatory shear stress leads to a pro-atherogenic environment with greater cathepsin activity and decreased cystatin C. Here, this project offers the novel idea that the upregulation of cathepsins and inverse downregulation of the cathepsin endogenous inhibitor, cystatin C, is a key point in blood vessel wall remodeling, atherosclerotic plaque development, and abdominal aortic aneurysm formation.

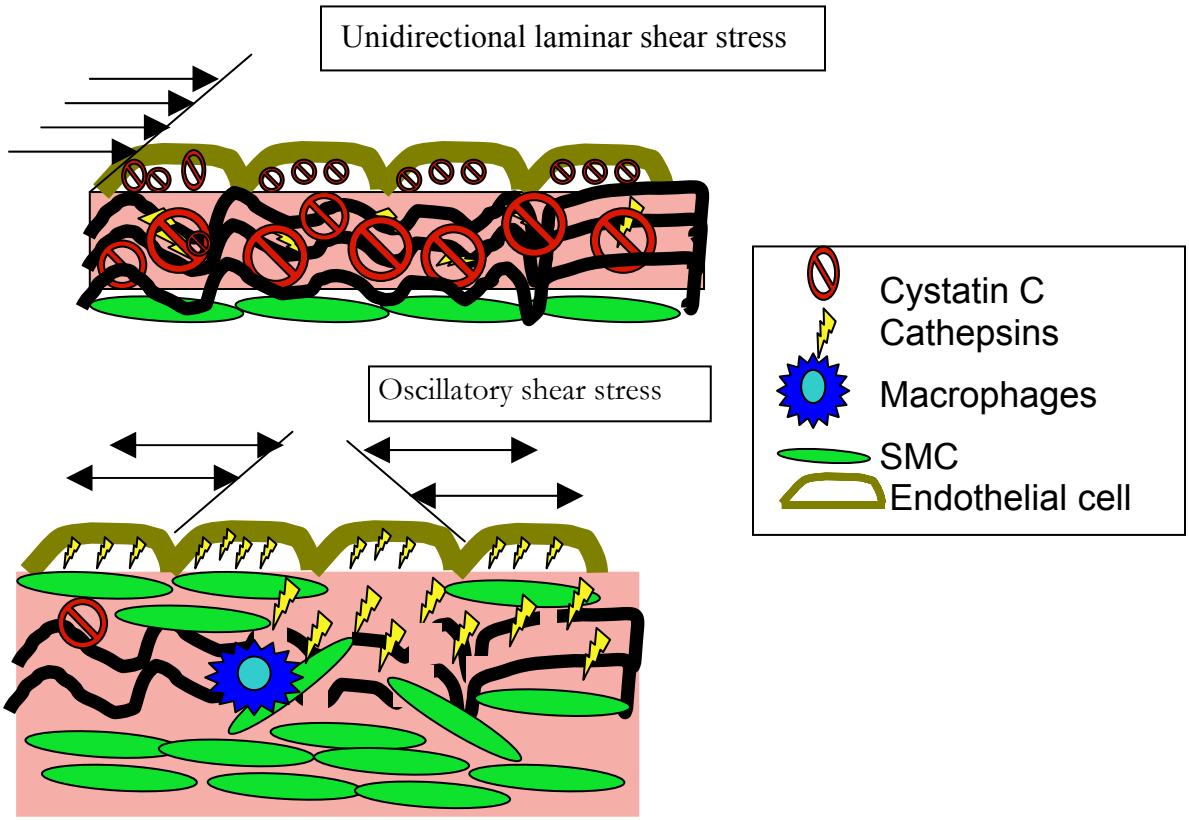

Figure 1.2: Schematic of overall hypothesis. Without the inhibition of cystatin C due to upregulation by unidirectional laminar shear stress, cysteine proteases can degrade elastic lamina, allow smooth muscle cells to migrate into the subintima, and atherosclerosis persists.

Specific Aim 1: Understand endothelial secretion and activation of cathepsins due to oscillatory shear stress and their contribution to extracellular matrix proteolysis.

Hypothesis: Oscillatory shear stress signals endothelial cells to secrete cathepsins, perhaps initially to maintain an open lumen and return shear stress level to normal. However, they become pathological once they persist and are uninhibited by cystatin C.

Specific Aim 2: Determine the atheroprotective, molecular role of cystatin C in the cardiovascular system in response to unidirectional laminar shear stress.

Hypothesis: Cystatin C is a mechanosensitive protein that serves to inhibit cathepsins from degrading the internal elastic lamina and remodeling the blood vessel wall in a unidirectional, laminar shear environment to retard atherosclerotic plaque development.

Specific Aim 3: Examine the alternative roles for cathepsins and cystatin C in endothelial cell physiology.

Hypothesis: Oscillatory shear stress turns on signaling pathways that lead to cathepsin transcription, expression, or activation in endothelial cells that may have other cellular functions besides protein turnover in lysosomes. It is important to understand these additional roles so that therapeutic interventions do not lead to other malfunctions in the cells.

The goal is to further understand how shear stress regulates proteolytic mechanisms in the blood vessel wall, specifically the cathepsin family and their inhibitor, cystatin. By understanding how the levels change, and how they are regulated, new therapeutic targets can be developed to retard plaque progression and aneurysmal formation and to save lives.

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CHAPTER 2

LAMINAR SHEAR STRESS INHIBITS CATHEPSIN L ACTIVITY IN ENDOTHELIAL CELLS

Introduction

Vascular endothelial cells are constantly exposed to fluid shear stress, the frictional force generated by blood flow over the vascular endothelium. The importance of shear stress in vascular biology and pathophysiology has been highlighted by the focal development patterns of atherosclerosis in hemodynamically defined regions. For example, the regions of branched and curved arteries exposed to disturbed flow conditions including oscillatory and low mean shear stresses (OS) correspond to athero-prone areas. In contrast, straight arteries exposed to pulsatory, high levels of laminar shear stress (LS) are relatively well protected from atherosclerotic plaque development (1).

Changes in blood flow have been shown to be a critical factor inducing arterial remodeling (1-7). Increases in arterial wall shear stress prevent vascular remodeling leading to thickening of the vascular wall and inflammation (2), while decreases in arterial wall shear stress promotes arterial remodeling and inflammation (2, 3). Additionally, low wall shear stress leads to degradation of the internal elastic lamina (IEL) (5). Despite these findings, the underlying mechanisms by which shear regulates proteases degrading vessel wall matrix and lEL are not well described. While there is a report demonstrating that shear regulates matrix metalloproteases (MMPs) in endothelial cells (8), it is not clear whether other proteases are also regulated by shear.

Cathepsins are the papain family of cysteine proteases which degrade elastin in addition to collagen (9). Unlike MMPs, the role for cathepsins in blood vessel

remodeling and cardiovascular disease has been understudied until recently. Cathepsins K, L, and S, potent elastinolytic proteases, have been identified in atherosclerotic plaques (10, 11) and in neointima following balloon angioplasty (12). Furthermore, cathepsins B, L and S also have been shown to be upregulated at the transcriptional level in the arteries of Apolipoprotein E-null mice fed an atherogenic diet (13). In addition, cathepsin activity is increased in abdominal aortic aneurysms (10, 14). Cathepsin L is classified as one of the potent mammalian collagenases and elastases (15-18) and is capable of cleaving mature, insoluble elastin (17). However, cathepsin L expression and its role in endothelial cells and atherosclerosis development are not well known.

Here, we hypothesized that shear stress regulates cathepsin activities in endothelial cells. We examined the effects of OS and LS on matrix proteolytic activities and cathepsin activity in endothelial cells. Our results show that LS reduces matrix protease activity in a cathepsin L-dependent manner.

Methods

Mouse Aortic Endothelial cells (MAEC) culture and shear stress studies. MAEC obtained from the thoracic aortas of C57/BL6 mice were isolated, cultured in growth medium [Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 100 μg/ml endothelial cell growth supplement (ECGS, Sigma) and 2.5 U/ml heparin] as described(19), and used between passages 7-10. Confluent endothelial monolayers grown in 100-mm tissue culture dishes were exposed to an arterial level of uni-directional LS (15 dyn/cm^2) or OS with directional changes of flow at 1 Hz cycle (± 5 dyn/cm^2) for 1 day by rotating a Teflon cone (0.5° cone angle) as described

previously(19). One hour prior to shear, the monolayers were washed and changed to 10 ml of fresh shear medium (the growth medium without serum).

Elastase and gelatinase assay. Five μg/ml of BODIPY® fluorescein-conjugated DQ™ elastin or gelatin (Molecular Probes) in 5 ml of fresh serum-free DMEM was incubated with MAEC following exposure to OS, LS, or St for one day in the presence or absence of the cathepsin inhibitor E-64 (Sigma). After an additional 24 hours, aliquots (200 μl) of conditioned media were assayed with a fluorescence plate reader, in triplicate, with background fluorescence subtracted from the no-cell negative control at 485 nm excitation and 525 nm emission.

Cathepsin zymography. Conditioned media were concentrated 20- to 30-fold with a spin concentrator (5kDa cutoff, Vivascience) and protein concentration was determined with a modified Lowry assay(20). Equal amounts of protein were resolved by 12.5% SDS-polyacrylamide gels containing 0.2% gelatin at 4°C. Proteins were renatured in 50 mM Tris buffer, pH 7.4 with 20% glycerol, and incubated overnight in assay buffer containing 0.1 M sodium acetate buffer, pH 5.5, 1 mM EDTA, and 2 mM dithiothreitol(21) in the presence or absence of cathepsin inhibitors: B (1 μM CA074), L (1 μM Z-FY(*t*-Bu)-DMK), K (1 μM 1,3-Bis(CBZ-Leu-NH)-2-propanone, Calbiochem), or S (1 nM Mu-Leu-Hph-VS-Ph)(22). Gels were then rinsed with deionized water, stained with Coomassie Blue and destained, and analyzed by densitometry.

Cysteine protease active site labeling. Conditioned media were normalized by volume and an equal aliquot (20 μl) was labeled with DCG-04 (5 μM), a biotinylated active site probe(23) (a gift from Dr. M. Bogyo) for 30 minutes before being boiled and resolved by a 12.5% SDS-PAGE. Blotted membranes were then probed for biotin with the VectaStain Elite kit (Vector Labs). Purified and denatured (boiled for 5 minutes) cathepsin L (Sigma) were used as positive and negative controls.

Cathepsin activity assay. Cells were lysed in 40 mM sodium acetate buffer, pH 5.5, .1% Triton-X 100, and conditioned media were collected and concentrated. Aliquots were added to a reaction mixture containing 100 mM acetate, pH 5.5, 2.5 mM EDTA, 2 mM dithiothreitol, and 0.1% Brij 35. Benzyloxycarbonyl-Arg-Arg-7-amino-4-methylcoumarin (Z-RR-AMC) (Biomol) was used as the substrate and added to attain a final concentration of 5 μM after the cathepsins were activated for two minutes at 37°C(24). The reaction mixture was incubated at 37°C for 10 minutes, and AMC fluorescence intensity was determined with a fluorescence plate reader (excitation at 360 nm and emission at 460 nm).

Western blots. Following shear, cells were lysed with RIPA buffer and conditioned media concentrated as above. Equal amounts of total protein were resolved by SDS-PAGE, and the blots were probed with antibodies to cathepsins L (1:500, R&D), K (1:200, Calbiochem), B (1:250, Calbiochem), and S (1:1000, Santa Cruz), or β-actin (1:1000, Santa Cruz) and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by a chemiluminescence method(20).

Transfection of siRNA: Sub-confluent (75-80%) MAEC were transfected with annealed siRNA duplex [sense: 5'-UCAUUGAGGAUCCAAGUCAtt, antisense: 5'-UGACUUGGAUC CUCAAUGAtt] or non-silencing duplex [sense: 5'-UUCUCCGAACGUGUCACGUtt, antisense: 5'-ACGUGACACGUUCGGAGAAtt] (Qiagen) using Oligofectamine (InVitrogen) in serum free medium. After 6 hours, the medium was supplemented with serum (final 10% concentration) and cultured an additional 18 hours prior to exposing the cells to OS, LS, or no flow conditions.

Statistical Analysis*:* Student's unpaired t-test was used to establish significance between groups. P<.05 was considered statistically significant.

Results

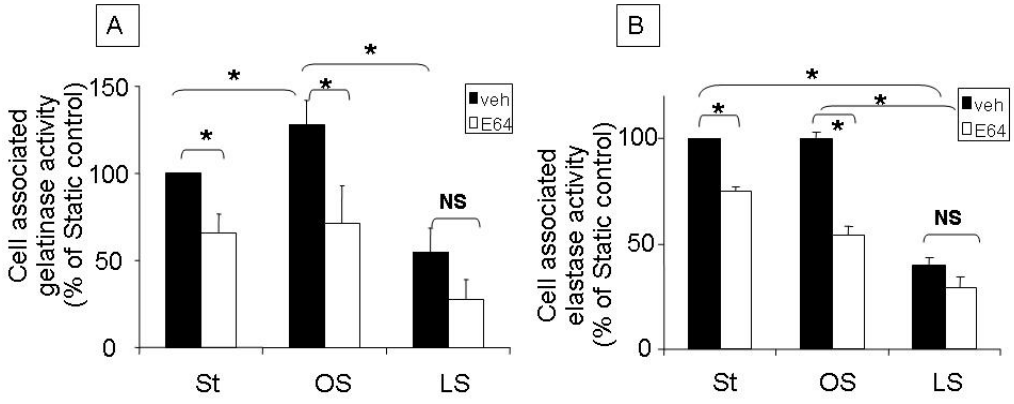

Figure 2.1. LS decreases cell-associated gelatinase and elastase activities in MAEC. A, B) Confluent MAECs were exposed to Static (St), oscillatory shear (OS), or laminar shear stress (LS) for 1 day in serum free medium. Following shear, BODIPY-elastin or -gelatin were added to intact cells and incubated overnight in fresh media with or without 50 μM E64. Gelatinase (**A**) and elastase (**B**) activities measured as an increase in fluorescence intensity (mean ± SEM, *=p<.001, n=5 to 14).

LS decreases cell-associated extracellular matrix proteolytic activity in endothelial cells.

To determine whether shear stress affected protease activities towards components of the extracellular matrix, we used BODIPY-gelatin and -elastin, soluble, fluorescently labeled gelatin and elastin, as matrix substrates to live endothelial cells that had been exposed to 24 hours of OS, LS, or no flow (static) conditions. LS exposure significantly lowered both gelatinase and elastase activities in MAEC in comparison to those of static and OS-exposure (Figure 2.1A and B). In contrast, OS exposure had mixed effects on the matrix protease activities: OS increased the gelatinase activity by 28% above that of the static control, but did not affect the elastase activity (Figure 2.1A and B). Next we examined how much of the total matrix protease activity was contributed by cathepsins using the cathepsin inhibitor E64. Treatment with E64 inhibited the gelatinase and elastase activities by 30 to 50% in the static and OS-exposed cells. On the other hand, E-64 did not have significant inhibitory effects on the gelatinase and elastase activities of LS-exposed cells (Figure 2.1A and B). These results raise a possibility that LS and E-64 target the same proteases, cathepsins that may be novel members of the mechanosensitive matrix proteases.

Endothelial cells exposed to LS have lower cathepsin activity than that of OS.

Next, we determined whether the mechanosensitive cathepsin activities are secreted into the conditioned media or remain associated with the cells. For this study, conditioned media were collected from MAEC that were exposed to OS, LS and static conditions for 1 day, and the cells were scraped and lysed to measure protease activity in

the lysate. The conditioned media and cell lysates were then used for three independent studies to determine cathepsin activities.

First, we carried out the gelatin zymography at an acidic pH and calcium-deficient environment, a condition that is optimum for cathepsins (21). The zymography using conditioned media and cell lysates obtained from static and OS-exposed cells revealed

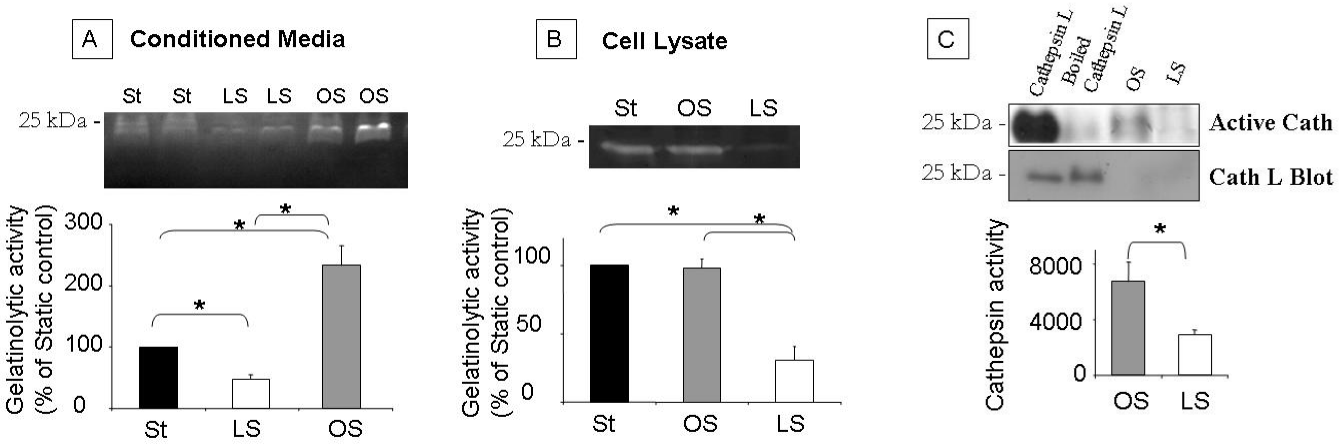

Figure 2.2. MAEC exposed to LS have lower cathepsin activity than that of OS. Following 1 day of exposure of MAEC to OS, LS, or static conditions as in Figure 1, conditioned media (A) and cell lysates (B) were collected, and equal protein amounts were assayed by gelatin zymography optimized for cathepsins. Representative zymograms show gelatinolytic activities at ~23 to25 kDa bands and densitometric quantification is shown in the bar graphs (mean ± SEM, n=7 to 9, *p< .05). **C**: Equal volume of the conditioned media were labeled with the biotinylated active probe DCG-04 (5 μM), resolved by SDS-PAGE, and the blot developed with a streptavidin-HRP method. Purified and denatured (boiled) cathepsin L were used as positive and negative controls, respectively. As an additional control, the blots were reprobed with a cathepsin L antibody. The biotin blot was quantified by densitometry as shown by the bar graph (n=3, p<.001).

visible bands with apparent molecular masses of 23 to 25 kDa, indicating the presence of active proteases (Figure 2.2A and B). In comparison, exposure of MAEC to LS significantly reduced the gelatinase activity in both the conditioned media and cell lysates (Figure 2.2A and B). On the other hand, OS exposure showed a 2.5-fold increase in the

gelatinase activity in the conditioned media (Figure 2.2A). This OS effect, however, was not observed in the cell lysate (Figure 2.2B).

Second, the active protease activity associated with the 23/25 kDa proteins found in the conditioned media was further confirmed by an independent assay using DCG-04, which binds to active cathepsins (23). As shown in Figure2C, the conditioned media obtained from OS exposed MAEC contained significantly higher amount of active cathepsins with ~25 kDa size in comparison to that of LS exposure. As positive and negative controls, the purified active cathepsin L and the inactive (boiled) enzyme were used. As expected, only active form of cathepsin L, but not the boiled enzyme bound to the DCG-O4 label (Figure 2.2C). Western blot of the same membrane showed that the amount of cathepsin L in the non-concentrated conditioned media of MAEC was not sufficient to be detected, although the DCG-O4 label clearly identified the enzymes. These results suggest that shear-sensitive cathepsin activity is secreted into the media as detected by the more sensitive assays (zymography and the DCG-O4 labeling study) although the Western blot study was not sensitive enough to show the enzyme identity.

Cathepsin L activity is regulated by shear stress in endothelial cells.

To further determine which cathepsin(s) was responsible for the shear-dependent matrix protease activity, we used four cathepsin inhibitors during the cathepsin gelatin zymography assay. Again, the conditioned media of MAEC exposed to LS contained significantly reduced gelatinase activity in comparison to that of OS and static cells (Figure 2.3A). The inhibitors of cathepsin B (CA074) and S (Mu-Leu-Hph-VS-Ph) had no effect on the gelatinolytic activity, while cathepsin K inhibitor [1,3-Bis(CBZ-Leu-NH)-2-propanone] showed a minor inhibitory effect (Figure 2.3A). The cathepsin L

inhibitor [Z-FY(*t*-Bu)-DMK], however, completely blocked the gelatinase activity of the conditioned media from both OS and static-exposed cells.

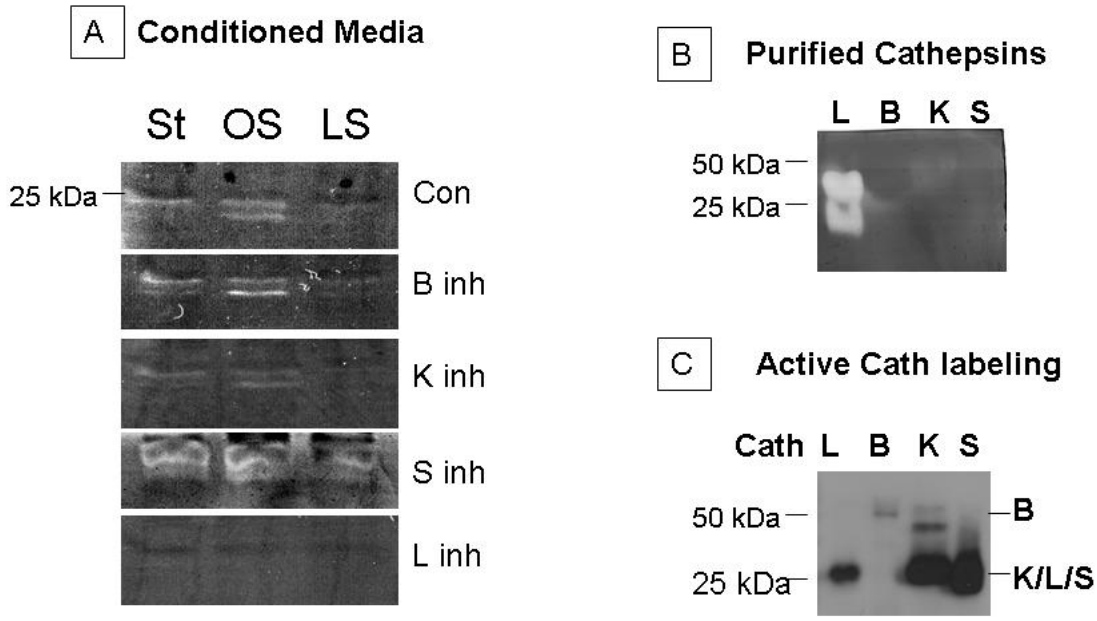

Figure 2.3. LS exposure inhibits cathepsin L activity in MAEC. A) Following 1 day of exposure of MAEC to OS, LS, or static conditions, conditioned media were collected and analyzed by the cathepsin gelatin zymography in the absence or presence of the inhibitors of cathepsin B (1 μM CA074), L (1 μM Z-FY(*t*-Bu)-DMK), K (1 μM 1,3-Bis(CBZ-Leu-NH)-2-propanone), or S (1 nM Mu-Leu-Hph-VS-Ph). Shown zymograms are representative of at least 3 separate experiments. Equimolar active amounts of purified cathepsins L, B, K, and S were used in cathepsin gelatin zymography **(B)** and DCG-04 active cathepsin labeling **(C)**.

In this gelatin zymography study, there was a possibility that the reason we observed only cathepsin L-like activity may have been due to a bias in assay conditions. For example, the zymography requires renaturation of the cathepsins following non-reducing SDS-PAGE. If for any reason cathepsins do not properly renature, we would not be able to detect their activities. To address this question, we loaded a gelatin gel with equivalent amounts of purified cathepsins L, B, K, and S based on their cathepsin activity assays using the peptide substrate Z-FR-AMC and E-64 titration curve (24). Of the four

enzymes, only cathepsin L, but not B, K and S, was capable of degrading the gelatin (Figure 2.3B). Active site labeling of the cathepsins showed that all four of the cathepsins were present in their active state (Figure 2.3C). These results show that the cathepsin zymography condition used in this study is sufficient for cathepsin L activity while the other cathepsins may not be active, possibly due to their failure to be renatured during the zymography assay. Based on these results, we cannot rule out whether cathepsins B, K, and S are mechanosensitive matrix enzymes or not. Nevertheless, the pharmacological results suggest that cathepsin L activity in the conditioned media is a mechanosensitive matrix protease.

Cathepsin L siRNA knocks down cathepsin L protein and significantly reduces endothelial gelatinase and elastase activity.

To definitively address whether cathepsin L is a shear-sensitive matrix protease, we used a siRNA approach. Treatment of MAEC with cathepsin L siRNA knocked down cathepsin L protein expression more than 80% below that of non-silencing controls in static, OS and LS-exposed cells as shown in Western blots of the conditioned media. To examine the specificity of the siRNA against cathepsin L, we immunoblotted the cell lysates with antibodies for cathepsins B, K, and S. Cathepsin L siRNA did not cause nonspecific knockdown of cathepsin K and S (Figure 2.4A). However, it reduced cathepsin B protein expression by ~50 %; cathepsin B exists as a 31 kDa single chain that is then processed into a 25/26 kDa double chain as reported by Linebaugh et al. (25)

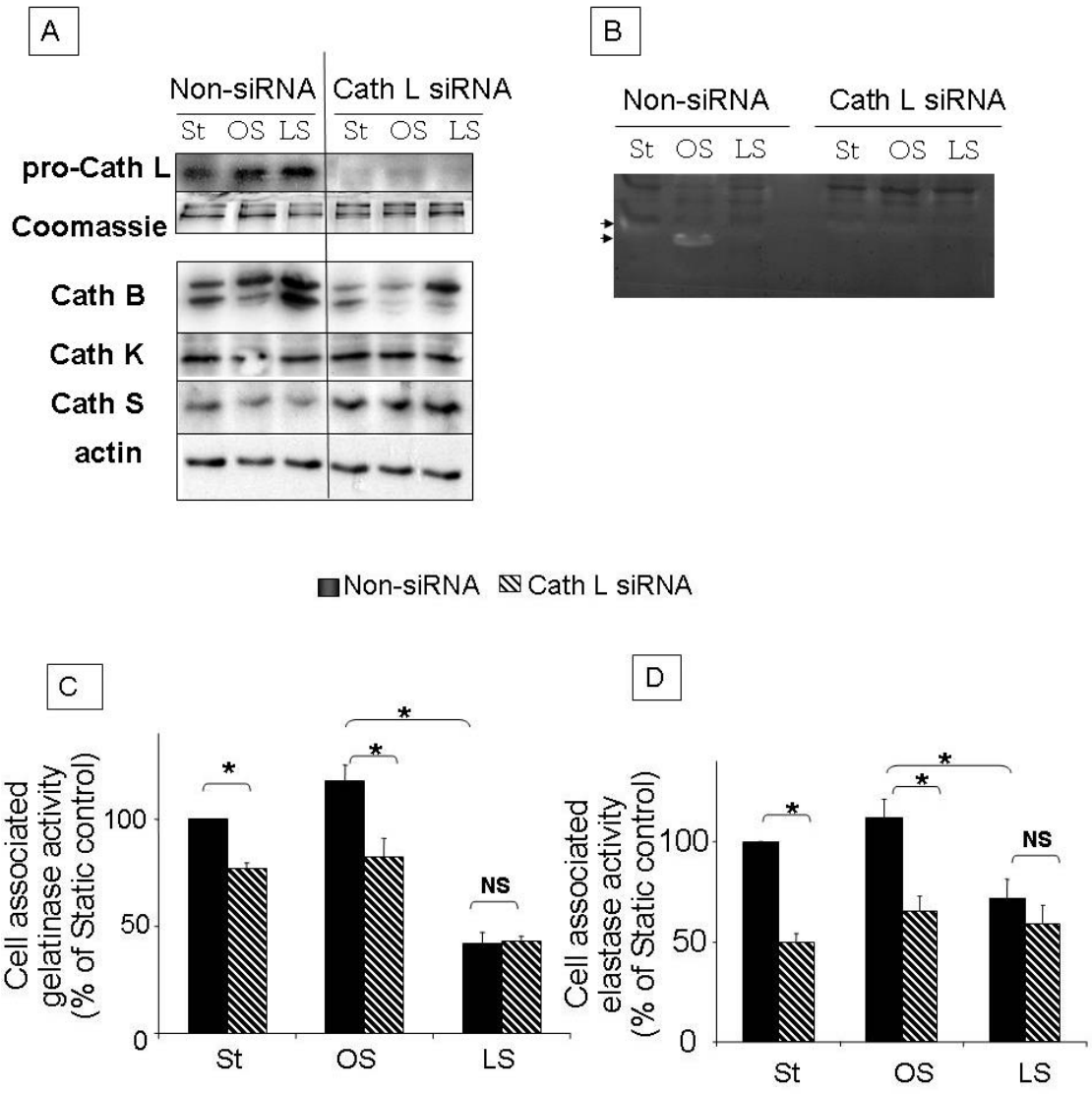

Figure 2.4. Cathepsin L siRNA knocks down cathepsin L protein and reduces endothelial cell-associated elastase activity. Sub-confluent MAECs were transfected with cathepsin L specific siRNA (100 nM) or non-silencing RNA (non siRNA) 24 hours prior to shear exposure. Transfected cells were then exposed to OS, LS, or static conditions for 1 day. **A)** Cathepsin L protein knockdown by cathepsin L siRNA was confirmed by Western blot using conditioned media and the cathepsin L antibody with Coomassie staining of the gel as a loading control. Cell lysates were collected and probed with antibodies to cathepsins B, K, and S, and a β actin antibody as an internal control. In **B**, conditioned media obtained from A were examined by gelatin zymography as in Figure 2. The arrows indicate cathepsin L bands. The cell-associated gelatinase **(C)** and elastase (**D)** activities were determined using BODIPY-gelatin and -elastin as described in Figure 1. Shown are mean ± SEM, (*p<.05, n=4). ns: p> 0.05

Under identical conditions, cathepsin L siRNA treatment of MAEC blocked the cathepsin L activity stimulated by OS in the conditioned media (Figure 2.4B). This result provides strong evidence that the shear-sensitive gelatinolytic activity detected by the zymography is indeed cathepsin L. Additionally, cathepsin L siRNA treatment of the cells significantly inhibited the static and OS-induced gelatinase and elastase activities as determined by the degradation of BODIPY-gelatin and –elastin. Cathepsin L siRNA significantly inhibited both gelatinase and elastase activities of static and OS groups, although it tended to show a greater inhibitory effect on the elastase activity than the gelatinase (Figure 2.4B vs. 2.4C). Cathepsin siRNA significantly reduced the gelatinase activity of static and OS groups by 25 and 30% of their non-silencing static and OS controls, respectively (Figure 2.4B). In a similar trend, the siRNA significantly inhibited the elastase activity by 50 and 40% of the non-silencing control static and OS groups, respectively (Figure 2.4C). In contrast, the cathepsin L siRNA had no significant effect on the gelatinase or the elastase activity in cells exposed to LS. These results are consistent with the E-64 results (Figure 2.1), suggesting that cathepsin L is an important shear-dependent matrix protease.

Shear stress does not affect cathepsin B activity in endothelial cells.

With the above cathepsin L siRNA results, there remained a question whether the reduction in the gelatinase and elastase activities by cathepsin L siRNA treatment was caused by an unexpected partial knockdown of cathepsin B. Therefore, we decided to examine whether cathepsin B is a shear-sensitive protease. For this purpose, we used the cathepsin B specific peptide substrate, Z-RR-AMC to assess cathepsin B activity. The

results showed that neither LS nor OS significantly changed cathepsin B activities from that of the static conditions in either the cell lysates (Figure 2.5A) or conditioned media (Figure 2.5B) obtained from MAEC. Next, we examined whether the cell-permeable cathepsin B inhibitor (CA074Me) would reduce the cell-associated elastase activity. For this study, we treated MAEC with CA074Me at 0.1 μM, a concentration that inhibits cathepsin B activity by ~70% (Figure 2.5C) without significantly affecting purified cathepsin L activity (data not shown). At this concentration, the cathepsin B inhibitor had no effect on the cell-associated elastase activities of the cells exposed to the static, OS and LS conditions (Figure 2.5D). These results not only show that cathepsin B activity is not regulated by shear stress, it also indicates that this enzyme does not play a critical role in the elastase activity associated with MAEC. Together, the results shown in Figures 2.3 and 2.4 strongly support the conclusion that the cathepsin L siRNA treatment inhibited the elastase activity in static and OS-exposed cells by knocking down cathepsin L expression.

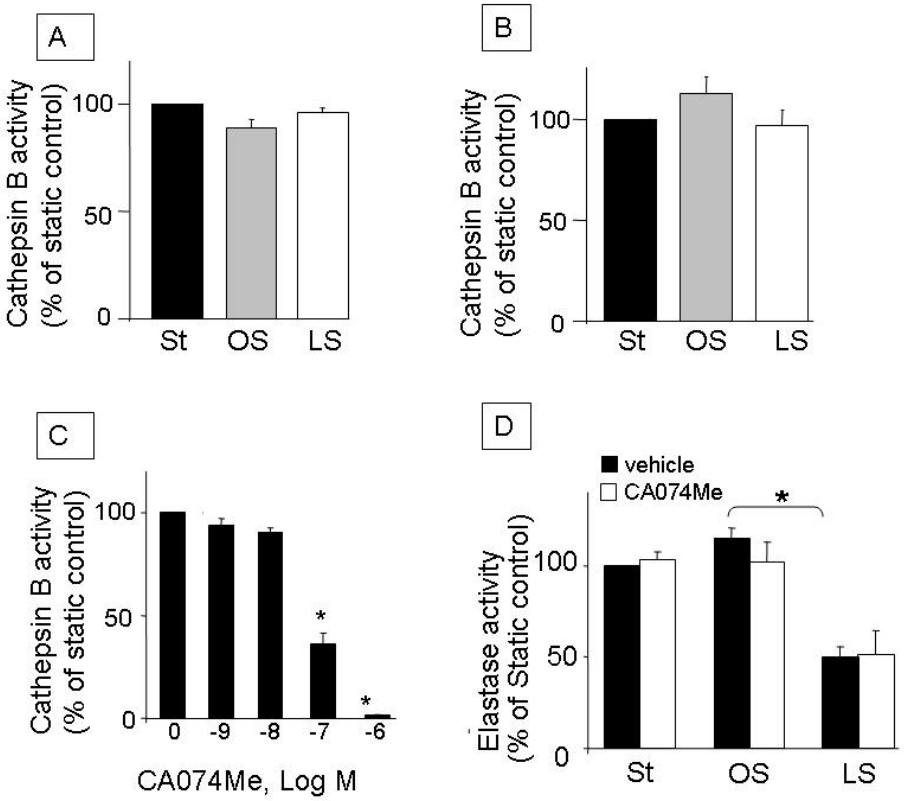

Figure 2.5. Cathepsin B activity is not affected by shear stress in MAEC. Equal protein aliquots of the cell lysates (**A**) and conditioned media (**B**) obtained from MAEC exposed to static, LS or OS for 1 day were incubated with the synthetic peptide substrate Z-RR-AMC specific for cathepsin B in acidic pH conditions. **C**. Static cell lysates were incubated with Z-RR-AMC as in A with the increasing concentrations of CA074Me. **D**. Following exposure of MAEC to static, OS, or LS for 1 day, cells were further incubated for 18 hr in fresh media containing BODIPY-gelatin with or without 0.1 μM CA074Me (mean ± SEM, *p< 0.05, n=4 to 6). ns: statistically not significant

Discussion

The novel and significant findings of this study are that: 1) exposure to atheroprotective LS decreases elastase and gelatinase activities of endothelial cells, 2) cathepsin L activity is inhibited by LS and is responsible in part for shear stress regulated matrix protease activity, and 3) cathepsin B activity is not regulated by shear stress and it does not significantly contribute to the elastase activity. These conclusions are

supported by several lines of evidence. Using fluorescent-gelatin and –elastin as substrates added to live cells, we have shown that LS exposure substantially inhibited their degradation compared to OS and static cultured cells, and that these proteolytic activities were inhibited by E-64. It should be noted that LS exposure lowered the protease activity to a minimum such that it could not be significantly further inhibited by a general cathepsin inhibitor E-64 or cathepsin L siRNA in this study. In addition, the gelatin zymography optimized for cathepsins and the active site labeling study with DCG-04 revealed that active cathepsins were produced and secreted in static and OS-exposed cells, while LS significantly reduced matrix proteolytic activities and cathepsin L, but not cathepsin B.

Evidence supporting the mechanosensitive regulation of cathepsin L was provided by the pharmacological and cathepsin L siRNA studies. The cathepsin B and S inhibitors had no effect on the gelatinolytic activity in the zymography using the conditioned media (Figure 2.2). Although it was partially reduced by the K inhibitor, the L inhibitor completely blocked all the activity, suggesting cathepsin L as the predominant proteolytic enzyme in that condition. It is important to note that the cathepsin zymography used in our study turned out to be effective for cathepsin L activity, but not for cathepsins K, B and S (Figure 2.2). Therefore, the partial effect of the cathepsin K inhibitor observed in Figure 3A is likely due to its non-specific effects on cathepsin L. We further suggest that cathepsin L is an enzyme responsible for the shear-sensitive gelatinolytic activity of the cell lysate and the conditioned media determined by the zymography (Figure 2.1).

The significant effect of cathepsin L siRNA in knocking down cathepsin L expression and gelatinase and elastase activities further supports its role as a

mechanosensitive protease. This interpretation was somewhat complicated by the unexpected effect of the siRNA on cathepsin B, requiring further studies. Importantly, however, our further studies revealed that cathepsin B activity did not change by LS or OS in MAEC, nor did it contribute to the elastase activity associated with the cells as shown by the lack of the cathepsin B inhibitor effect on it. These results again support the conclusion that cathepsin L, but not cathepsin B, is an important shear-sensitive matrix protease in endothelial cells.

The role for cathepsins and their inhibitor cystatin C in elastic lamina degradation, vascular remodeling and atherosclerosis has been demonstrated in animal models and humans. Mice deficient in cathepsin S in LDLR-null mice show decreased internal elastic lamina (IEL) fragmentation and reduction in atherosclerosis (26). Cystatin C deficiency in ApoE-null mice resulted in increased elastic lamina fragmentation and collagen content, which could have contributed to the dilation of thoracic and abdominal aortas (27). It remains controversial whether cystatin C deficiency affects atherosclerosis (28, 29). Increased cathepsin L expression and decreased cystatin C have been found in human atherosclerotic plaques and aortic aneurysms (10, 30).

The differential effects of laminar and oscillatory shear stresses on cathepsin L activity reported in this study may be a critical mechanism by which AAA occurs in regions of disturbed flow. Several human and animal studies have demonstrated that atherosclerotic lesions and aneurisms of the abdominal aorta occur in the regions where they are exposed to unstable flow conditions including flow reversal, low mean wall shear stress and high oscillatory shear index (31-35). In contrast, relatively high levels of laminar shear stress were shown to reduce AAA progression in rat experimental

models(36) and a recent report found increased cathepsin L levels in human AAA but almost no detectable cathepsin L in normal arteries (10). Together, these previous findings and our current study raise an interesting possibility that differential regulations of cathepsin L by undisturbed and disturbed flow conditions may play a critical role in the protection or initiation and progression of AAA.

Shear stress potently regulates vascular remodeling, including the sizes of lumen and IEL fenestrae (3, 6, 7, 37), and flow-dependent arterial remodeling is endothelium-dependent(3). While the mechanisms controlling cathepsin activities in smooth muscle cells (11) and macrophages (22, 38, 39) have been reported, the role of cathepsins in endothelial cells have been rather limited. Shi et al showed that cathepsin S deficiency led to abnormal angiogenic responses due to abnormal extracellular matrix degradation (39). Also, cathepsin L has been shown to play an important role in endothelial progenitor cell mediated neovascularization (37). While shear stress has been shown to increase cathepsin B activity in neutrophils, (40) the current study is the first report showing that cathepsins are regulated by shear stress in endothelial cells. The reason that cathepsin B is shear-sensitive in neutrophils but not in endothelial cells as we showed here may be due to unique cell-specific responses.

Shear stress has been shown to regulate another family of matrix proteases, MMP's both in cultured endothelial cells and in animal models. OS, but not LS, significantly stimulates MMP-9 mRNA and protein expressions in murine lymphoid endothelial cells (8). Consistent to that report, we also found that OS stimulated MMP2/9 activities in MAEC as measured by gelatin zymography (data not shown). Using an arterio-venous fistula model in wild-type and knockout mice, flow-induced vascular

remodeling has been shown to involve MMP activity that is mediated by the NADPH oxidases and nitric oxide-dependent mechanisms (41). In a rabbit model using a carotid branch ligation method, low flow was shown to upregulate MMP-2 and MMP-9 (42) .

Shear stress regulates structure and function of endothelial cells and plays an important role in atherosclerosis development. The atheroprotective LS may protect the integrity of elastic laminae and extracellular matrix by inhibiting cathepsins such as L, while the pro-atherogenic OS have opposite effects. In summary, we showed that cathepsin L is a mechanosensitive matrix protease with a potential importance in vascular remodeling and atherosclerosis.

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CHAPTER 3

ROLE OF CATHEPSIN L IN ENDOTHELIAL CELL ALIGNMENT AND ELONGATION

Introduction

Endothelial cells line the arterial wall and are directly exposed to the fluid shear stress of the blood as it drags along the wall. This shear stress elicits several responses in the endothelial cell, including elongation and alignment in the direction of flow, presumably to reduce the local mechanical load and injury. At sites of disturbed flow such as bifurcations and sharp turns where there are regions of flow separation and recirculation, the endothelial cells remain unaligned; this has been demonstrated both *in vivo* and *in vitro*. Several signaling proteins and pathways are involved in endothelial alignment including mechanosensory complexes to detect the flow and its direction (1), actin and microtubule cytoskeletal components to reorient the cell (2), calcium influx (2), and integrin activation to establish new focal adhesions and signaling complexes (3, 4). Microtubule disruption (2) and inhibition of p38 mitogen activated protein kinase (5) both block endothelial cell alignment. It has recently been shown that vascular endothelial cadherin, platelet endothelial cell adhesion marker-1, and vascular endothelial growth factor receptor 2 were sufficient to induce alignment in the direction of flow when transfected into COS 7 African green monkey cells that do not normally have this response (1).

Formation of new integrin-ligand complexes have also been shown to be upstream of Rho signaling and to participate in EC alignment with flow (3). Establishing new integrin-ligand complexes may also involve remodeling of matrix. Alpha V beta 3 integrin has increased activation and affinity in response to shear stress

(3) and blocking it with a peptide antagonist reduces flow-mediated vasodilation (6). This integrin is also functional in angiogenesis and tumor metastasis, serving to bind MMPs at the cell surface in migrating cells (7). It also binds mature cathepsin S, which is capable of degrading several extracellular matrix components, on the surface of vascular smooth muscle cells (8).

Integrin dissociation and reattachment to extracellular matrix components may also involve breakdown and reorganization of basement membrane matrix, but protease involvement in endothelial cell alignment and elongation has not been investigated. An initial finding that knockdown of cathepsin L with siRNA inhibited EC elongation and alignment while performing other studies, led us to develop the hypothesis that cathepsin L is involved in alignment of endothelial cells. We show here that knockdown of cathepsin L with siRNA but not pharmacological inhibitor blocks EC alignment and elongation and also alters p38 and ERK signaling mechanisms in the endothelial cell.

Methods

Mouse Aortic Endothelial cells (MAEC) culture and shear stress studies. Endothelial cells obtained from the thoracic aortas of C57/BL6 control were isolated and cultured in growth medium growth medium [DMEM containing 20% fetal bovine serum (FBS), 100 μg/ml endothelial cell growth supplement (ECGS, Sigma) and 2.5 U/ml heparin)] as described previously (9) and used between passages 7-10. Confluent endothelial monolayers grown in 100 mm tissue culture dishes were exposed to an arterial level of uni-directional LS (15 dyn/cm^2) or OS with directional changes of flow at 1 Hz cycle (± 5 dyn/cm^2) by rotating a Teflon cone (0.5° cone angle) as described previously by us (10)

One hour prior to shear, the monolayers were washed and changed to 10 ml of fresh shear medium (the growth medium without serum).

Western blots. Following shear exposure, conditioned media were collected and normalized to 10 ml total with fresh serum free shear media if necessary, and concentrated 20 to 30 fold with a spin concentrator (5 kDa molecular weight cutoff, Vivascience). Cells were rinsed twice with phosphate buffered saline, lysed with RIPA buffer, and sonicated briefly. Following modified Lowry protein assay, equal amounts of total protein were resolved by SDS-PAGE. Protein was transferred to a polyvinyldifluoride membrane (Millipore), and probed with a rabbit polyclonal anti-phosphorylated p38, total p38, ERK 42/44, phosphorylated ERK 42/44, JNK, and phosphorylated JNK (1:1000, Cell Signaling) and a secondary antibody conjugated to alkaline phosphatase (Bio-Rad), which were detected by a chemiluminescence method (11).

Transfection of siRNA*:* Sub-confluent (75-80%) MAEC were transfected with annealed siRNA duplex [sense: 5'-UCAUUGAGGAUCCAAGUCAtt, antisense: 5'-UGACUUGGAUC CUCAAUGAtt] or non-silencing duplex [sense: 5'-UUCUCCGAACGUGUCACGUtt, antisense: 5'-ACGUGACACGUUCGGAGAAtt] (Qiagen) using Oligofectamine (InVitrogen) in serum free medium. After 6 hours, the medium was supplemented with serum (final 10% concentration) and cultured an additional 18 hours prior to exposing the cells to OS, LS, or no flow conditions.

Phalloidin staining. Cells were rinsed with PBS, and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS, and the fixative was quenched with 50 mM ammonium chloride. Blocking buffer of 3% BSA in PBS was preincubated with the cells for an hour, then incubated with rhodamine-phalloidin (1:100) dilution. Cells were rinsed with PBS then covered with Prolong anti-Fade Kit (Molecular Probes) and visualized on an epifluorescent microscope.

Alignment and elongation calculation. Images of four different fields were captured of either the light micrograph, or the fluorescent image of the phalloidin labeled cells. LSM Image software (Zeiss) was used to draw a line in the direction of the stress fibers seen in the phalloidin labeled cells to determine its angle with the direction of flow. Randomly aligned cells approach an orientation angle of 45°, and aligned cells' angle goes to 0°. For elongation, the software was used to measure the cell perimeter and the area of the cells. The cell shape index was calculated according to the equation 4π x cell area/(cell perimeter)$^{2.}$ As the cells elongate, the shape index goes to zero but it goes to 1 if they are perfect circles (5).

Statistical Analysis. Student's unpaired t-test was used to establish significance between groups. $P<.05$ was considered statistically significant.

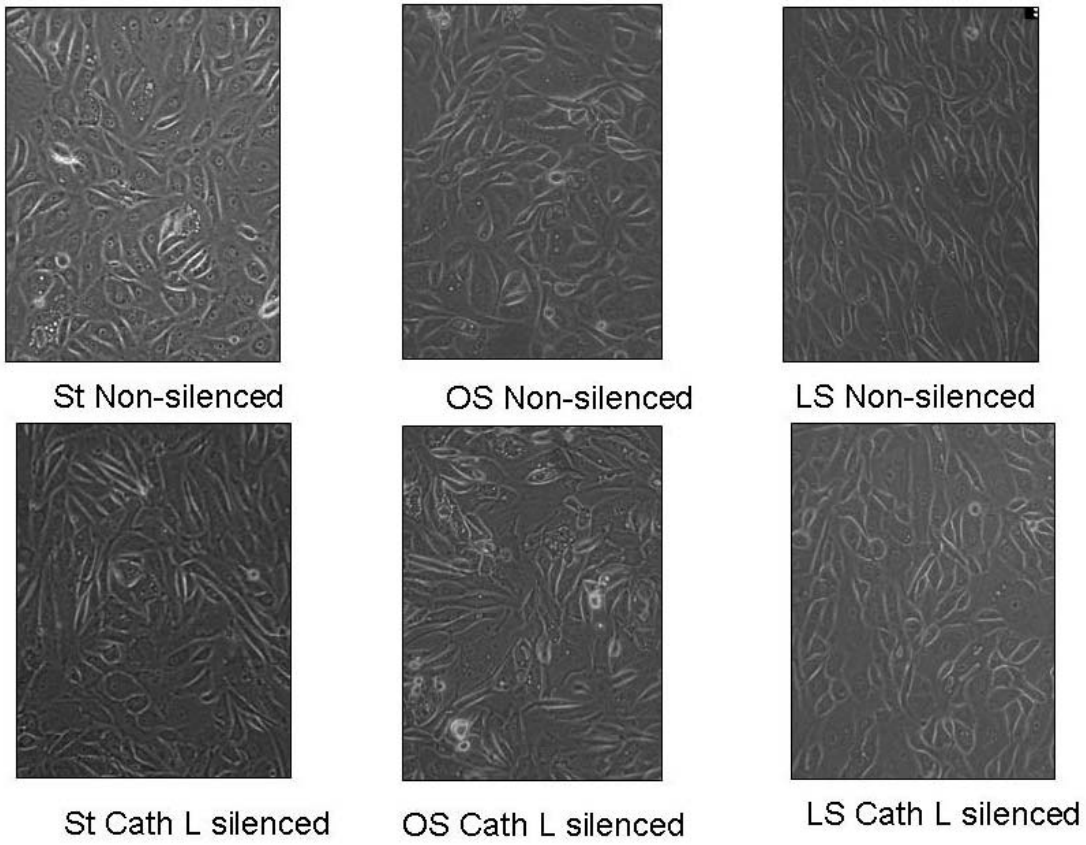

Figure 3.1. Cathepsin L siRNA blocks endothelial cell alignment in response to LS. Confluent MAECs were exposed to 24 hours of OS, LS, or St conditions. Representative micrographs are shown.

Knocking down cathepsin L blocks endothelial cell alignment and elongation in response to laminar shear stress.

In our previous studies using cathepsin L siRNA to study ECM proteolytic activity (12), we found that the endothelial cells transfected with cathepsin L siRNA did not elongate and align in response to LS (Figure 3.1) but those transfected with a nonsilencing RNA do. This illustrated that the transfection protocol was not causing this phenomenon but led us to question whether the loss of cathepsin L was responsible.

Transfection with cathepsin K siRNA does not block this morphological change (Figure 8.1). We repeated this experiment and quantified endothelial cell alignment and elongation. MAEC were transfected with cathepsin L siRNA 24 hours prior to exposure to LS or no flow conditions for 24 hours. Then digital images of the cells were captured (Figure 3.2). Other cells were fixed and stained with rhodamine-phalloidin to visualize the actin cytoskeleton. Alignment to direction of flow and the index of elongation were calculated as described (5). MAEC with cathepsin L knocked down showed a significantly higher cell shape index indicating no elongation and also had a significantly higher angle of alignment illustrating they are not aligning with the direction of flow (Figure 3.3).

Since cathepsin L has been demonstrated by us to play a role in degradation of ECM (12), we hypothesized that reduction in that activity with siRNA led to a loss of remodeling ability of the ECM and as a consequence, alignment. We used a cathepsin L pharmacological inhibitor to test this as well. Surprisingly, the MAEC still elongated and aligned in the presence of 10 μM of the cathepsin L inhibitor, which did not support the hypothesis or the cathepsin L siRNA data.

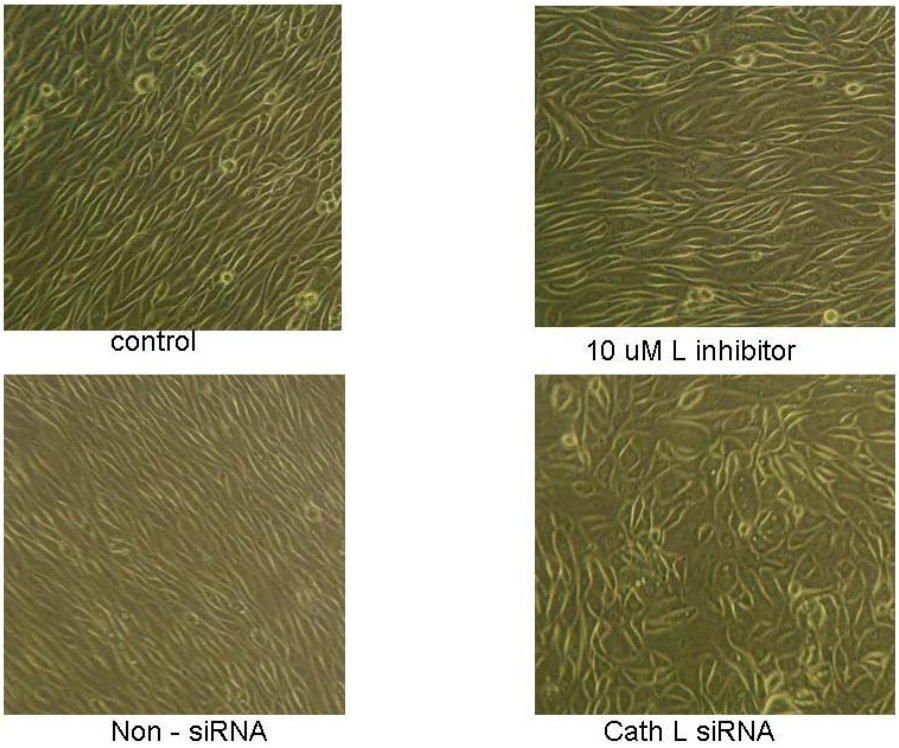

Figure 3.2. Cathepsin L inhibitor does not block endothelial cell elongation and alignment in response to LS. MAECs were transfected with non-siRNA or cathepsin L siRNA, or were incubated with cathepsin L inhibitor or vehicle, and exposed to 24 hours of LS. Representative micrographs are shown.

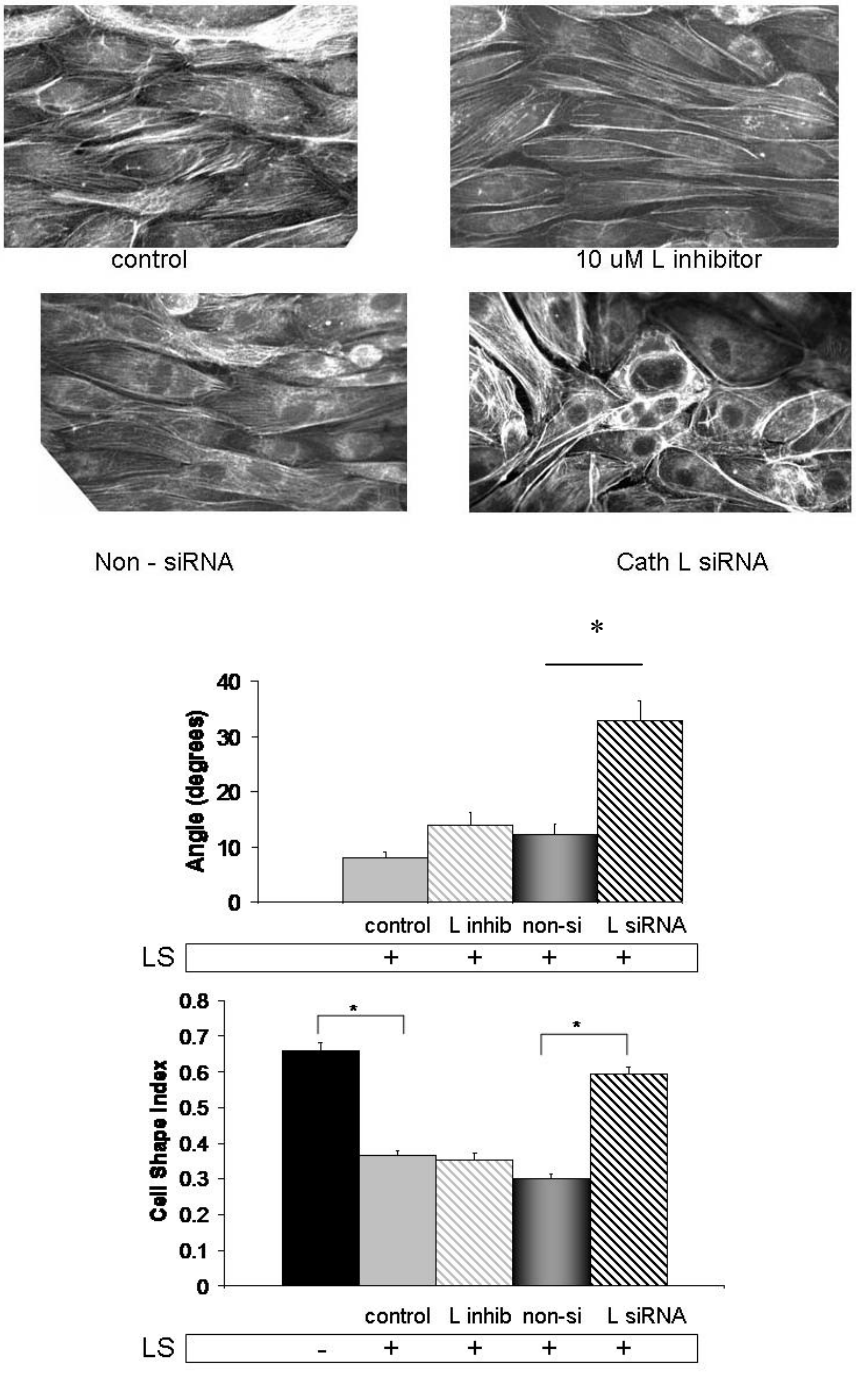

Figure 3.3. Cathepsin L inhibitor does not block endothelial cell elongation and alignment in response to LS. MAECs were transfected with non-siRNA or cathepsin L siRNA, or were incubated with cathepsin L inhibitor or vehicle, and exposed to 24 hours of LS and then fixed and stained with Rhodamine-phalloidin to visualize the F-actin structures. Representative micrographs are shown. Cell alignment and elongation were calculated as described in the methods from the phalloidin stained images (n=40, *p<.05).

To expand on the finding that cathepsin L knockdown impeded matrix remodeling for morphology change, we used a scratch wound assay to determine the role of cathepsin L in EC migration. Under static conditions, EC were transfected with cathepsin L siRNA 24 hours prior to replacing medium with serum free medium and scratching the confluent layer of cells. Cells were incubated for 18 hours and digital images were captured to view cell migration into the wounded area. Knocking down cathepsin L inhibited EC migration into the wound pathway as seen in Figure 3.4.

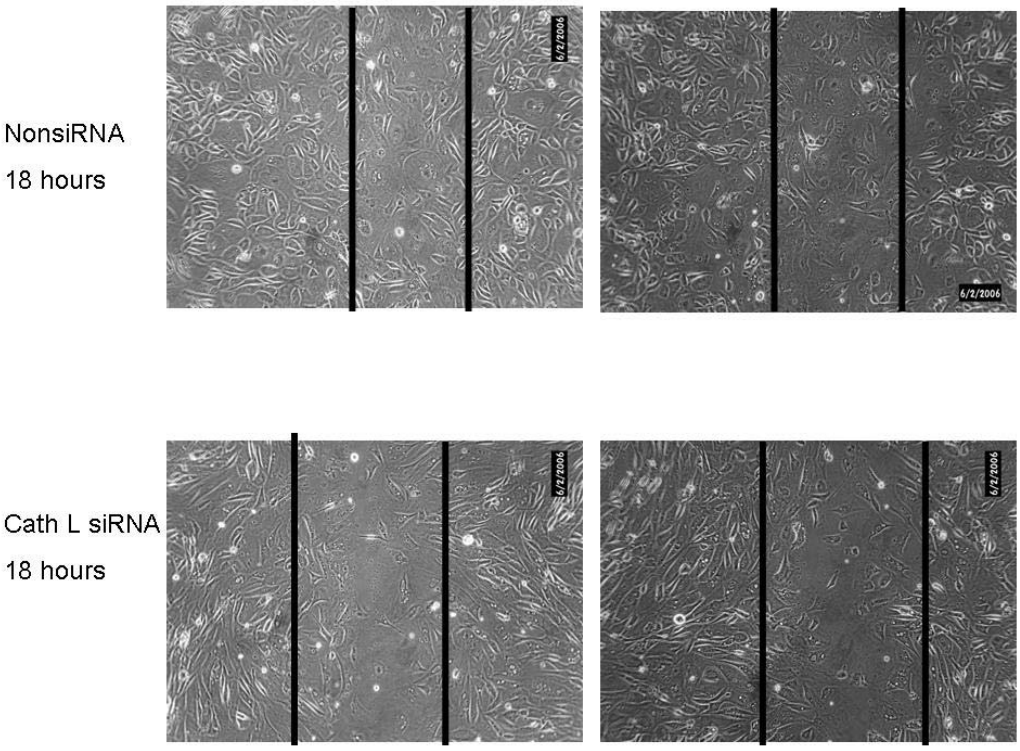

Figure 3.4. Knockdown of cathepsin L inhibits MAEC migration after scratching. Static culture MAECs were transfected with cathepsin L siRNA 24 hours prior to scratching the monolayer. Images were captured and compared to 18 hours later. Bars indicate original edges of the scratch wound. Images shown are representative of two independent experiments.

Cathepsin L knockdown and MAP kinases

Cathepsin L has not been reported as playing a role in EC migration or alignment so it became important to find a mechanism. Sumpio's group reported that blocking p38 MAPK with a pharmacological inhibitor blocked EC alignment in bovine aortic endothelial cells using a pharmacological inhibitor. We investigated if p38 was upstream or downstream of cathepsin L in shear mediated EC alignment. MAEC were transfected with either cathepsin L siRNA or nonsiRNA and exposed to LS or no flow for 24 hours. There was an elevated baseline phosphorylation of p38 after knocking down cathepsin L both under no flow conditions and after 24 hours of LS exposure (Figure 3.5 A, C).

MAP kinases are known to be involved in several signaling pathways, some of which are activated by shear stress in short time periods on the scale of minutes. This immediate activation leads to downstream effects of transcription and translation of key proteins. We examined the short term activation of p38 in response to LS and if knocking down cathepsin L would alter this time course of activation. MAEC transfected with cathepsin L siRNA or nonsiRNA were sheared for 0, 15, 30, 60 minutes or 24 hours, lysed and probed for p38 phosphorylation. Peak p38 activation was seen after 30 minutes of LS in those cells transfected with the non-silencing control, but knockdown of cathepsin L induced an earlier activation after only 15 minutes of LS (Figure 3.5A). There was also a higher baseline elevation of p38 phosphorylation at 0 hours and 24 hours of LS (Figure 3.5 A, C). Extracellular signal regulated kinase (ERK) has been studied in endothelial cells in response to shear stress and been linked to various shear stress signaling pathways. Baseline ERK activation was also increased in the MAEC with reduced cathepsin L levels at 0 hours and 24 hours of LS similar to p38 (Figure 3.5B).

An earlier peak activation of ERK occurred as well after 15 minutes of shear stress instead of the 30 minute time point seen in the control MAEC. JNK phosphorylation was not altered by transfection with cathepsin L siRNA (Figure 3.5D).

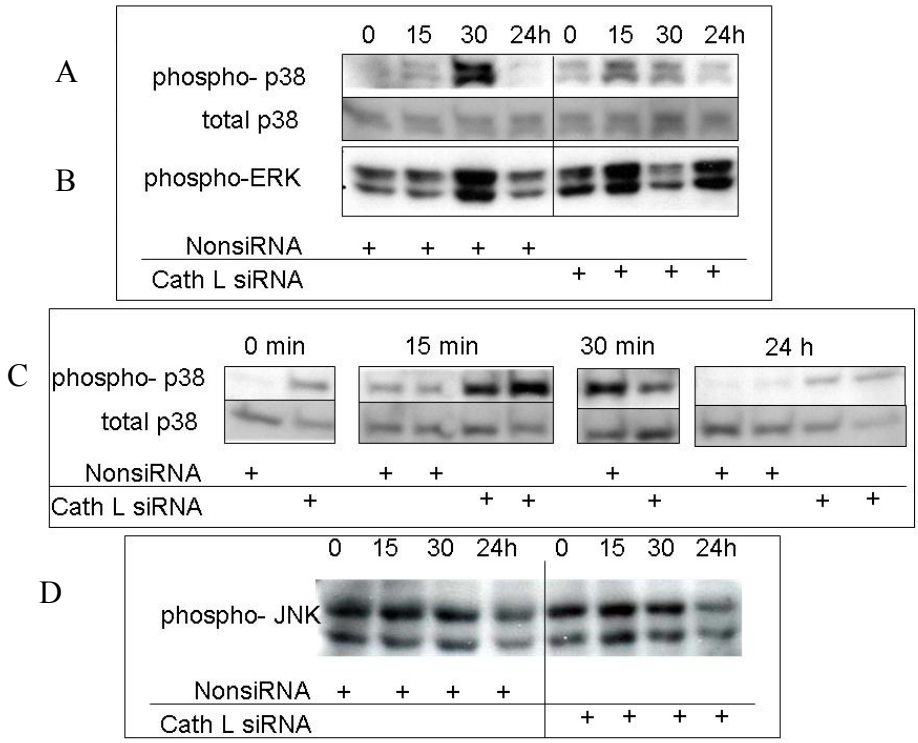

Figure 3.5. Knockdown of cathepsin L alters peak phosphorylation of MAP kinases. MAECs were transfected with either cathepsin L siRNA or nonsiRNA and exposed to LS for 0 min, 15 min, 30 min, or 24 hours. Cell lysates were probed for phosphorylation of p38 (A, C), ERK (B), and JNK (C). Blots shown are representative of at least two independent experiments.

Discussion

These results illustrate a role for cathepsin L in endothelial cell alignment and elongation. Novel findings here are that (1) knockdown of cathepsin L with siRNA blocks EC alignment and elongation in response to laminar shear stress; (2) Cathepsin L knockdown inhibits endothelial cell migration; and (3) knockdown of cathepsin L leads

to elevated p38 and ERK activation but does not affect JNK suggesting that p38 and ERK are involved either upstream or downstream of cathepsin L dependent EC migration.

Rearrangement of ECM proteins underlying endothelial cells occurs in response to laminar shear stress (13), and cathepsin L is capable of degrading these matrix components necessary for remodeling of the basement membrane and subsequent cell alignment and migration. Molecular biological knockdown of cathepsin L with siRNA affected EC alignment, but the cathepsin L inhibitor had no significant effect. Chapman et al showed cells can create intimate microenvironments for enzyme function that are not penetrable by soluble inhibitors using alveolar macrophages that were capable of degrading insoluble fibrin whether the cells made contact with it or not in the absence of proteinase inhibitors. However, in the presence of the inhibitors, the macrophages could still degrade the fibrin but only when in contact with it (14). Here, we saw that the molecular biological knockdown of cathepsin L with siRNA could block the EC alignment but the soluble inhibitor did not; it could be that the siRNA reduces the protein level so that there is no cathepsin L to work at the microenvironment, whereas, with the addition of the inhibitor, functional cathepsin L is present in the cell, but the sequestration of the inhibitor from the functional site of matrix remodeling allows the cells to elongate and align.

Cathepsins have been canonically referred to as the lysosomal cysteine proteases, but much research has revealed their involvement in other cellular processes both intra- and extra-cellularly. Particularly, cathepsin L has been shown to have a surprising role in the cell nucleus where it cleaves a transcription factor CDP/cux that serves to regulate the cell cycle (15). To this end, we conclude that cathepsin L is playing a role in endothelial

cell alignment with the direction of flow and have observed the signaling mechanisms involved after cathepsin L knockdown implicating p38 MAPK and ERK 1/2. Early activation of these kinases in response to shear stress leads to downstream events later, and both have been shown to regulate cathepsins in other systems. p38 MAPK has been shown to be essential for expression of cathepsin K in maturing osteoclasts after RANKL induction (16).

Tumor studies of cathepsin L implicate the Ras- Raf- MAPK/ERK(MEKs) – ERK1/2 pathway in cathepsin L expression and activation where constitutively active ras expression led to increased protein levels of cathepsin L in fibroblasts through the Raf/ERK pathway which was necessary (17). Sustained ERK activation by the cell adhesion molecule L1 increased alpha V beta 3 integrin, cell motility, and cathepsin L, again linking ERK activation and cathepsin L (18). Increased ERK activation increased cathepsin L dependent motility of transformed NIH 3T3 fibroblasts with decreased JNK activity, but when there was increased JNK activity and decreased ERK activity, the proteolysis switched to urokinase plasminogen dependent motility (19). Knockdown of cathepsin L in our system causes ERK and p38 to be turned on, but JNK is not.

Cathepsin L contribution to the ECM remodeling that occurs during EC alignment and migration deserves further investigation to understand the proteolytic mechanisms that take place under flow conditions. Our earlier findings show that endothelial cells actually decrease the activity of cathepsin L intracellularly and extracellularly under LS, but there may be a basal level of cathepsin L that is used at either integrins or focal adhesions to facilitate migration and alignment. Co-localization and co-immunoprecipitation studies will be useful in the future towards determining this role.

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CHAPTER 4

CYCLIC PRESSURE AND SHEAR STRESS REGULATE MATRIX METALLOPROTEINASES AND CATHEPSIN ACTIVITY IN PORCINE AORTIC VALVES

Introduction

Heart valve disease is a serious condition that affects a significant percentage of the population both in the U.S and worldwide. According to the American Heart Association statistics, valvular diseases cause nearly 20,000 deaths per year and are related to another 42,000 deaths. Current surgical interventions include valve repair or replacement depending on the diagnosis. Significant progress has been made during the last century on the development and improvement of prosthetic valves; however, so far there is no ideal replacement valve available. Recent advances in tissue engineering have spurred the interest in heart valve tissue engineering. The success of a tissue engineered heart valve (TEHV) will rely heavily on the understanding of native valve biology, which unfortunately, has only been studied very limitedly.

Native heart valves, the aortic and the mitral valves in particular, function in a harsh and complex mechanical environment. The valvular structure constantly responds to the surrounding mechanical environment. Close correlations between mechanical stresses and heart valve biology have long been documented by clinical observations and animal studies (1-3). According to these studies, the structural components of the aortic valve undergo constant renewal in response to mechanical loading (1), and the sites of protein and glycosaminoglycan synthesis in the leaflets correlate with the functional stresses (2). Similarly, changes in mechanical loading

alter biosynthetic behavior of valve cells. For instance, collagen synthesis in mitral valve leaflets was enhanced as a result of altered stress distribution due to left ventricular infarctions (4). Although, it is obvious that mechanical factors play a very important role in heart valve biology, so far, there is a paucity of literature concerning this subject. These studies have shown that cultured porcine aortic valvular cells responded to applied shear stress in a very unique way (5). Moreover, the mechanotransduction pathway seems to be different from the extensively studied vascular cell counterparts (5, 6). Previous studies in our laboratory showed that elevated pressure, both steady and cyclic, caused enhanced ECM synthesis in porcine aortic heart valve leaflets (7, 8). It is clear from these studies that mechanical factors such as pressure and shear stress are important in valve ECM remodeling; however, the molecular mechanisms remain largely unexplored. An increase in ECM synthesis accompanied by a decrease in ECM degradation may be induced by reducing enzyme activity of MMPs and cathepsins or by regulating their activity through protein inhibitors of these proteases.

Cathepsin L is a member of the papain family of lysosomal cysteine proteases, first identified in lysosomes, but has been also found to have functions in other cellular compartments, including the nucleus (9), and even extracellularly in different disease conditions such as arthritis (10, 11) and cancer (12). Intracellularly, cathepsin L is a powerful protease involved in protein turnover; extracellularly, cathepsin L is a powerful collagenase and elastase necessitating its tight regulation by pH, reducing environment, and protein inhibitors. In the cardiovascular system, cathepsin L has been found to be active within abdominal aortic aneurysms and atherosclerotic

lesions (13), while the absence of cathepsin L in knockout mice causes dilated cardiomyopathy (14) once again showing that although initially found in lysosomes, this protease is involved in other aspects of physiology. We have recently shown that steady laminar shear inhibits cathepsin L activity in mouse aortic endothelial cells (15). Other cathepsins have been linked to cardiovascular disease as well, particularly cathepsins S and K, which have been linked to elastic lamina degradation during atherosclerosis plaque development (16, 17), as well as in myxomatous heart valves (18).

The MMP family of proteases is another large member family that has been studied in the context of vascular remodeling (19-21) and in valvular remodeling (18, 22, 23) .

In the current study, we examined the hypothesis that MMPs and cysteine-dependent cathepsins are regulated by the mechanical environment, particularly pressure and steady laminar shear stress. We exposed the fresh porcine aortic valve leaflets to the highest pressure tested (mean of 170 mmHg, 1.167 Hz), and steady laminar shear stress of 25 dyne/cm^2. These were the same pressure and shear stress conditions used in our previous studies on valve ECM synthesis (7, 8).

Methods

Tissue collection and culture. Fresh porcine aortic valves were obtained aseptically from a local slaughterhouse (Holifield Farm, Covington, GA) and transported to the laboratory in ice-cold sterile Dulbecco's Phosphate Buffered Saline (DPBS) (BioWhittaker, Walkersville, MD). The three leaflets were cut from the annulus and

were bisected at the mid-line. One half was subjected to cyclic pressure or steady shear stress, and the other half was exposed to atmospheric pressure to serve as a control. Each experiment was run for 48 hrs, with a sample size ranging from 6 to 10 leaflets.

Cyclic pressure and shear stress experiment. Valve tissue was put in 12-well tissue culture plates and placed in an in-house designed polycarbonate pressure chamber (7, 8). The tissues were exposed to pressure conditions (150-190mmHg, at a frequency of 1.167 Hz) for up to 48 hrs. For shear stress studies, valve leaflets were mounted onto a modified parallel plate (24), with the ventricular surface facing flow. Steady laminar shear stress of 25 dyne/cm^2 was applied onto the leaflets for 48 hrs. Control leaflet tissues were placed in 12 well plates with 3 ml DMEM in each well. They were incubated under static conditions at 37^{o}C, 5% CO_2 and atmospheric pressure.

Gelatin zymography and immunoblot. Following mechanical exposure, half of a porcine aortic valve leaflet tissue was solubilized in lysis buffer (in mmol/L: Tris-HCl 20 (pH 7.5), EGTA 5, NaCl 150, glycerol-phosphate 20, NaF 10, sodium orthovanadate 1, 1% Triton X-100, 0.1% Tween 20) (20) and centrifuged at 14,000 rpm. Supernatant (lysate) was assayed for protein concentration with modified Lowry assay and equal amounts of protein (20μg) were loaded into 12.5% SDS-PAGE with 0.2% gelatin and electrophoresed at 4°C. Proteins were renatured in 50 mM Tris buffer, pH 7.4 with 20% glycerol for cathepsins or 2.5% Triton-X for

MMPs, and incubated overnight in assay buffer containing 0.1 M sodium acetate buffer, pH 5.5, 1 mM EDTA, and 2 mM dithiothreitol (DTT)(25) with or without the presence of 1 μM E-64, the cathepsin inhibitor or in 50 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$, 50 mM NaCl, and 0.05% Triton X-100 for MMPs. Gels were then rinsed with deionized water and stained with Coomassie.

Aliquots of lysates were also loaded into 12.5% SDS-PAGE without gelatin and transferred to a polyvinyldifluoride (PVDF) membrane. Membranes were probed with a goat anti-cathepsin L primary antibody (R&D) and then with a rabbit anti-goat secondary antibody conjugated to alkaline phosphatase. CDP-Star reagent was added and chemiluminescence was detected with photographic paper.

Cathepsin activity assay. Remaining half of the porcine aortic valve leaflet tissue was homogenized in a 0.15 M NaCl solution and centrifuged at 14,000 rpm for 15 minutes followed by modified Lowry protein assay on the supernatant. Activity assay procedure has been modified from that previously published(26): Brij 35 at 0.1% (Sigma) was added to 25 μg of total protein in a final concentration of 100 mM acetate, pH 5.5, 2.5 mM EDTA, 2 mM DTT for two minutes at 37°C to activate cathepsin L. Then Z-FR-AMC (Biomol), a synthetic substrate susceptible to cathepsin L hydrolysis, was added to a final concentration of 5 μM for ten minutes prior to reading AMC fluorescence released in a 96-well plate with excitation at 360 nm and emission at 460 nm.

Immunohistochemistry. Leaflet tissues were snap frozen in liquid nitrogen for cathepsin immunohistochemistry (IHC). The orientation of the leaflet was controlled by holding the tissue upright so that the cross section contained the three layers while placing it in the viscous media (OCT), and then the block was quickly dipped into liquid nitrogen. The block was cut into 7μm sections and stored under –80^{o}C until staining. Separate leaflets were embedded in paraffin and sectioned for MMP IHC. Frozen sections were thawed at room temperature and then immersed in acetone for 5 minutes. The slides were then washed in PBS twice, 5 minutes each, followed by immersing in 0.3% H_2O_2 for 15 minutes to block the endogenous peroxidase. After this step, the slides were incubated in 2% BSA/PBS for 15 minutes to suppress non-specific binding. The blocking step was followed immediately by primary antibody incubation (anti-cathepsin L, dilution of 1:20, Santa Cruz, S6501, anti-MMP-2, dilution of 1:50 (Calbiochem) or anti-MMP-9, dilution of 1:50 (Calbiochem) for 1 hour followed by 2 washes in PBS, each for 5 minutes. Next was the secondary antibody incubation (anti-goat IgG, 1:400) for 30 minutes, followed by 2 washes in PBS and then incubation in ABC-peroxidase (Vector Lab) for 1 hour. Subsequently, the slides were incubated in DAB (Vector Laboratories, SK-4100, Burlingame, CA) until color developed, the exact time varies with the amount of antigen in the tissue, but total development time was less than 10 minutes. Slides were then washed in water for 5 minutes and counterstained with hematoxylin. Cytoseal 60 (Richard Allan Scientific) was used as a resinous mounting agent, and the slides were covered and allowed to dry overnight before viewing with a Nikon Eclipse E600w microscope.

Results

Cyclic pressure inhibits cathepsin L and MMP2/9 activity in aortic heart valves.

Aortic heart valve leaflets were cultured for 48 hours under 170 mm Hg cyclic pressure before homogenization. Gelatin zymography showed a 37% decrease in cathepsin activity under cyclic pressure in comparison to that of the static control (Figure 4.1B). The cathepsin inhibitor, E64, completely blocked the gelatinase activity, demonstrating it is cathepsin activity. The decrease in cathepsin activity was not due to the change in cathepsin L protein level as shown by Western blotting with the specific antibody (Figure 4.1A). MMP2/9 activity showed a minor, but significant decrease of 17% by cyclic hypertensive pressure (Figure 4.1C). Fresh aortic valve leaflets contained minimal proMMP-2 activity and some cathepsin L activity (Figure 4.1D).

Cathepsin L is expressed throughout the aortic valve leaflet.

We next examined whether cyclic pressure affects cathepsin L expression in the aortic valve leaflets and its location. As shown by the IHC staining with the cathepsin L antibody in Figure 4.2, cathepsin L protein was diffusely expressed in the spongiosa, ventricularis, and fibrosa regions when cultured under atmospheric (Figure 4.2A) or cyclic pressure conditions (Figure 4.2B).

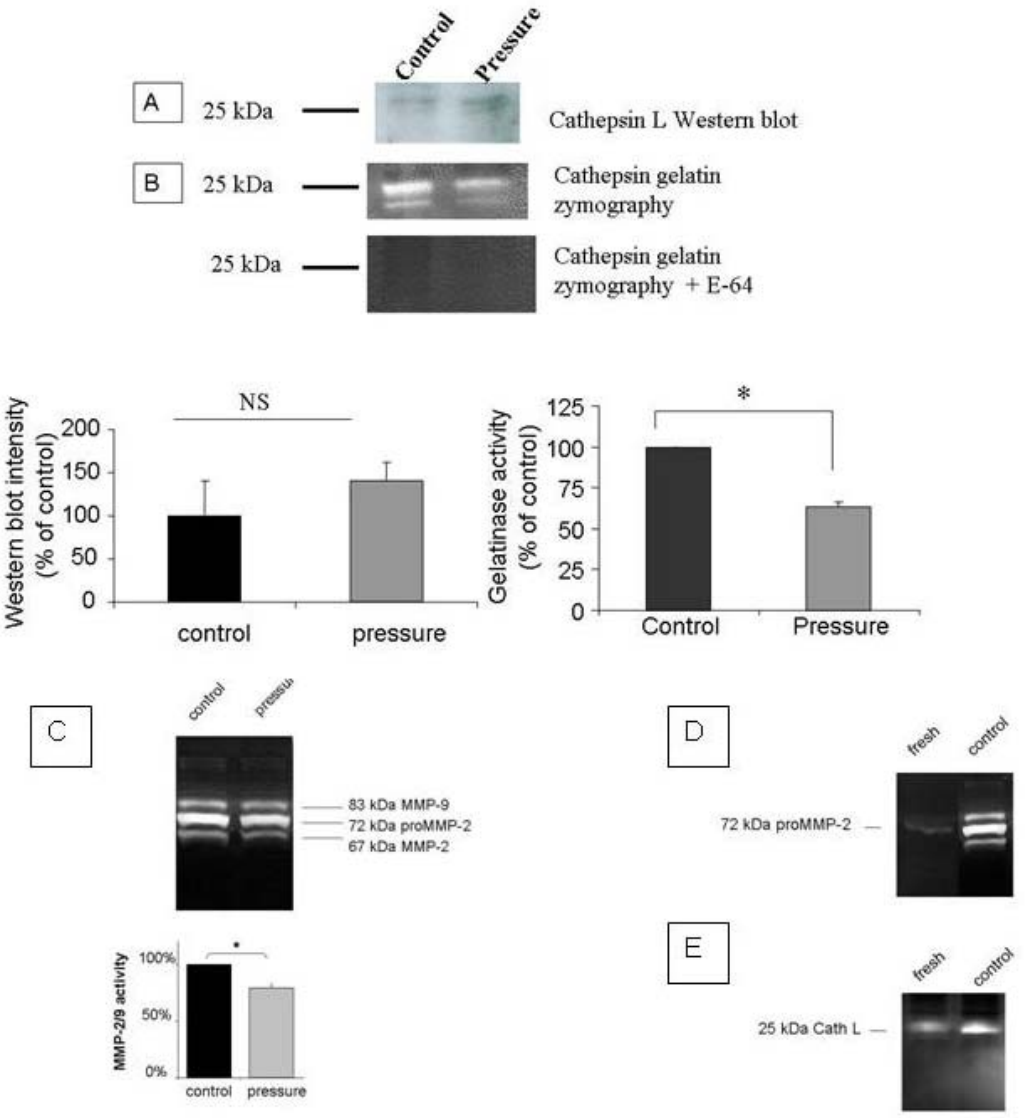

Figure 4.1. Cyclic pressure decreases cathepsin L activity in aortic valve leaflets. Fresh porcine aortic valve leaflets were exposed to hypertensive pressure (cyclic: 1.167 Hz, 170 mmHg) or control (atmospheric pressure) for 48 hrs. Equal protein amount of leaflet lysates was analyzed by western blotting with the cathepsin L antibody (A) and gelatin zymography for cathepsins B) or for MMPs (C). Shown in A and B are a representative Western blot and gelatin zymography in the absence (upper) or presence (lower) of E-64. The bar graph shows gelatinase activity as determined by densitometric quantitation of the Western blot and zymogram (mean $\pm$ SEM, n=4, * $p<.05$). Gelatin zymography from fresh aortic valve leaflets for MMP-2/9 (Figure 1D) and cathepsin L (Figure 1E).

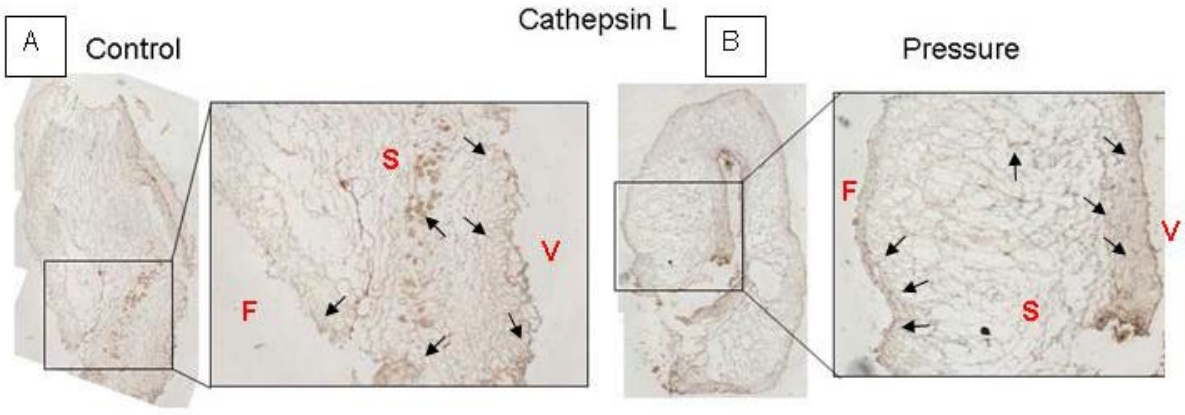

Figure 4.2: Cathepsin L is present in all layers of the aortic valve leaflet. Pig valve aortic leaflets that were exposed to cyclic pressure (170 mm Hg at 1.17 Hz) or static controls for 48 hr were frozen sectioned and immunostained with the cathepsin L antibody and counterstained with hematoxylin and eosin. V indicates the ventricular side of the leaflets. Light microscopic images were taken using a 4X objective. Cathepsin L expression is indicated by the brown staining and arrows. Shown is a representative image from 4 leaflets.

Aortic valve leaflets express MMP-2 more in the ventricularis than the spongiosa. MMP-2 expression was stronger in the ventricularis region of the aortic valve leaflets under atmospheric (Figure 4.3A) and cyclic pressure (Figure 4.3B) conditions. MMP-9 was distributed similarly (Figure 4.3C, D).

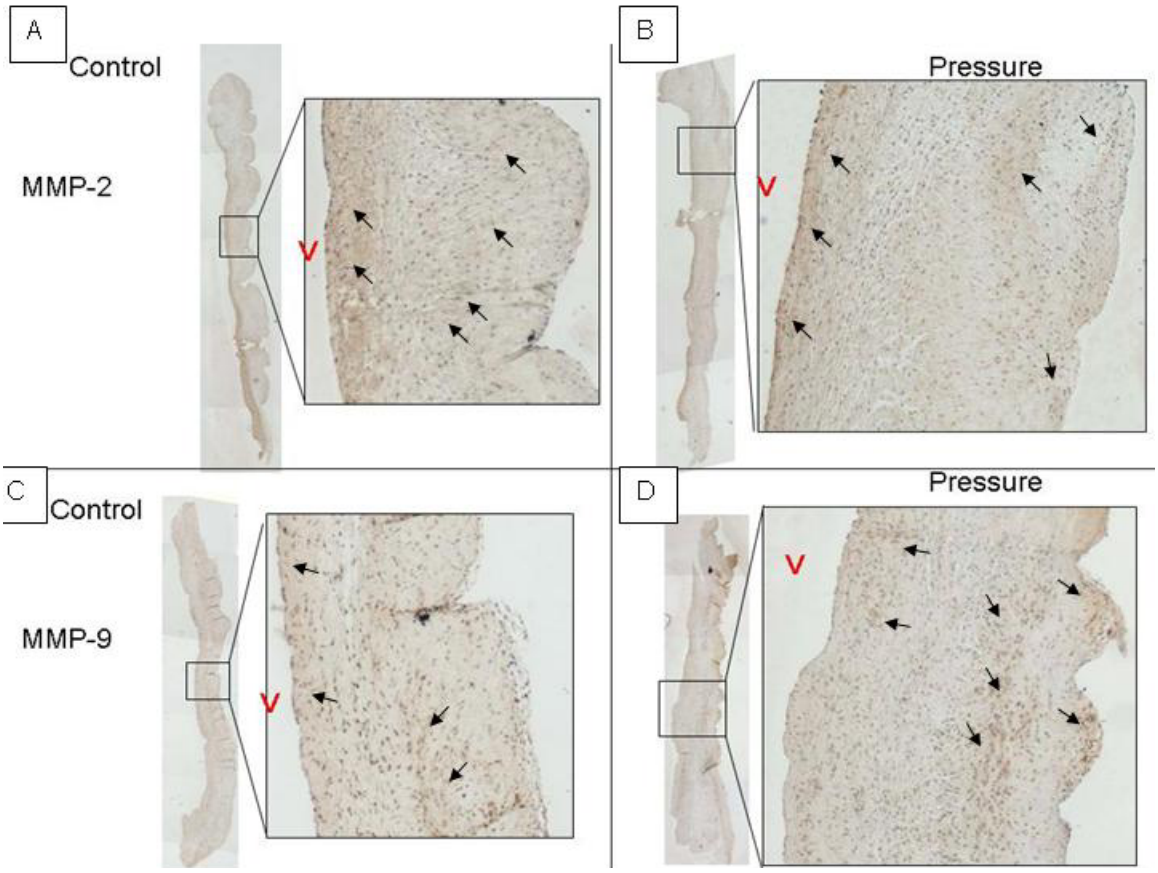

Figure 4.3: MMP-2 and MMP-9 immunohistochemistry on aortic valves under static conditions and cyclic pressure. Pig valve aortic leaflets that were exposed to cyclic pressure (170 mm Hg at 1.17 Hz) or static controls for 48 hr were paraffin embedded, and immunostained with the MMP-2 (A, B) or MMP-9 (C, D) antibodies. V indicates the ventricular side of the leaflets, and arrows indicate positive staining. MMP-2 staining is stronger in the ventricularis than other layers. Shown are representative light microscopic images from 4 different leaflets each.

Steady shear stress inhibits cathepsin L protein amount and activity.

Next, we examined the effect of laminar shear stress on cathepsin activity and cathepsin L protein expression. Exposure to 25 dynes/cm^2 shear stress for 48 hours decreased cathepsin L protein amount as seen by Western blot of porcine aortic heart valves (Figure 4.4A). In addition, shear stress significantly inhibited cathepsin activity as determined by two independent assays, gelatin zymography (Figure 4.4B) and cathepsin peptide substrate assay (Figure 4.4C). The cathepsin-specific activity

was demonstrated by the complete inhibition of gelatin proteolysis by E-64 (Figure 4.4B), and the inhibition of shear-induced cathepsin L-like activity is further supported by the synthetic substrate Z-FR-AMC, which has a preferential specificity toward cathepsin L (Figure 4.4C). In contrast to this, shear stress increased the activity of MMP2/9 in the aortic valve leaflets as seen by gelatin zymography (Figure 4.4D).

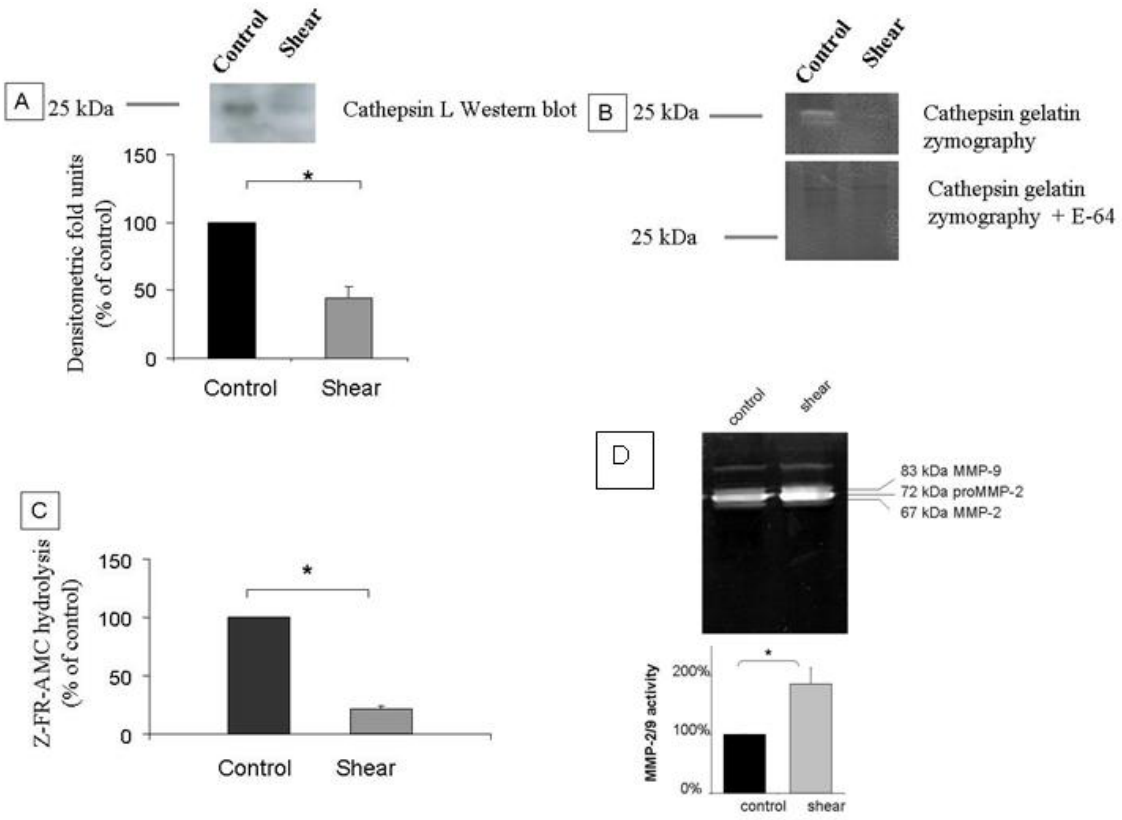

Figure 4.4: Steady shear stress decreases cathepsin activity and cathepsin L protein expression but increases MMP-2 activity in aortic valve leaflets. Porcine aortic valve leaflets that were exposed to steady laminar shear stress (25 dyne/cm^2) or no flow (as static control) for 48 hrs were frozen and lysed. Equal aliquots of lysates were analyzed by Western blot with cathepsin L antibody (A) and gelatin zymography for cathepsins (B) or for MMPs (D). Representative blots (A) and zymography (B) in the absence (upper) or presence (lower) of E-64, the cathepsin inhibitor, are shown. The bar graph is the densitometric analysis of Western blots or zymography (mean±SEM, n=4, *p<.05). In C, lysates were used to determine cathepsin activity using the synthetic peptide (Z-FR-AMC) substrate (mean±SEM, n=4, *p<.05).

We further examined in which area of the valve leaflet cathepsin L expression was affected by laminar shear by immunohistochemical staining with the cathepsin L antibody. As shown in Figure 4.5A, cathepsin L was diffuse in the leaflet under static culture but stains stronger in the ventricularis after exposure to steady laminar shear stress (Figure 4.5B). Overall, MMP-2 and -9 staining in the leaflets showed no remarkable differences between control and shear groups, but there was stronger signal in the ventricularis region compared to the spongiosa (Figure 4.6A-D).

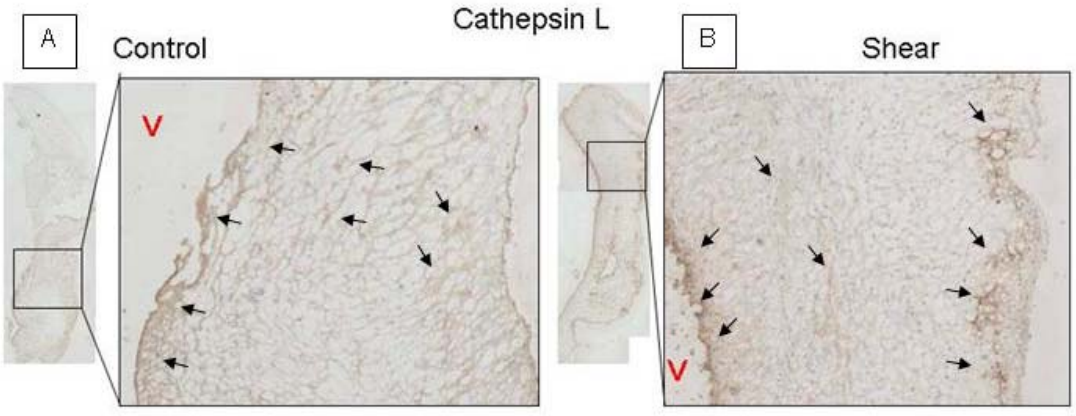

Figure 4.5: Cathepsin L immunohistochemistry in response to shear stress. Cathepsin L immunohistochemistry staining in leaflets exposed to shear stress was carried out as described in Figure 4.2. V- indicates the ventricular side of the leaflets, and arrows indicate positive staining. Note that the distribution of cathepsin L was different between shear and control, too. Under shear, majority of the staining was found in the ventricularis; while in the control, cathepsin L was diffusely distributed in the ventricularis and the spongiosa (n=3).

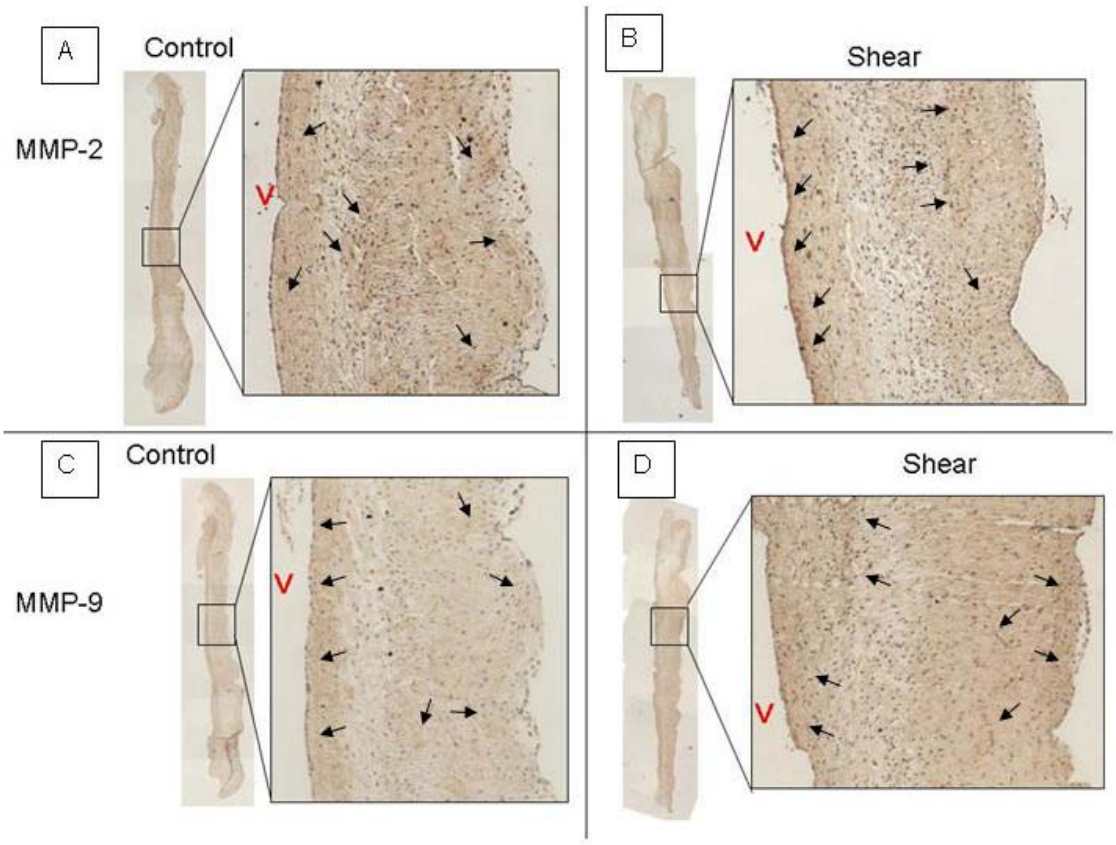

Figure 4.6: MMP-2/9 immunohistochemistry in response to shear stress. MMP-2/9 immunohistochemistry staining in leaflets exposed to shear stress was carried out as described in Figure 2. V- indicates the ventricular side of the leaflets, and arrows indicate positive staining. Under control and shear conditions, strong staining was found in the ventricularis (n=5).

Discussion

We have shown in the present study that hypertensive pressure caused a significant reduction in cathepsin L activity and a minor reduction in MMP2/9 activity in porcine aortic valve leaflets, while steady laminar shear stress decreased cathepsin activity and cathepsin L expression but increased MMP2/9 activity; our previous results showed significant increases in collagen content under identical mechanical conditions (7, 8). It appears that there is an inverse relationship between collagen synthesis and cathepsin activity under the stimulation of hypertensive

pressure or steady shear stress. Since the function of cathepsins are to degrade collagen, the observed higher collagen content under hypertensive pressure/shear stress conditions is likely due, in part, to a downregulation of the collagenolytic activities. The net collagen amount in the valve tissue is determined by a dynamic balance between new synthesis and degradation by collagenases such as cathepsins and MMPs.

The novel finding of the current study is that the cathepsins, collagenolytic proteases, are regulated by mechanical factors. Both, an increase in synthesis and a decrease in degradation will lead to higher total collagen content in the leaflet tissue. Previously, we showed that cathepsin L is inhibited by steady shear stress in aortic endothelial cells (15), but here, for the first time, we report that cathepsin L expression is regulated by cyclic pressure and shear stress and appears to correlate with the ECM renewal/remodeling in normal heart valve tissue. Previous studies have indicated the involvement of cathepsin K and S in myxomatous mitral valves (18), but so far there is no report on cathepsins in normal healthy heart valves. In addition to cathepsin L, we also studied MMP-2 and MMP-9 protein content and gelatinase activity because of their well-known role in ECM remodeling (27, 28) as well as in valve pathology (23, 29-31). We saw similar changes of MMP-2 and -9 induced by hypertensive pressure but opposite results with steady shear stress in the valve tissue. These two protease families may play different roles in ECM remodeling under shear stress; for example, one family could be responsible for degradation of the old components while the other is important in organization of the newly synthesized matrix.

In contrast to the shear effect, cyclic pressure inhibited cathepsin L activity without altering its protein level. This raises a possibility that cyclic pressure may regulate cathepsin activity either by controlling the cathepsin inhibitors such as cystatin C, or by directly inhibiting the cathepsin L activity through post-translational modifications.

The IHC staining results showed that cathepsin L was expressed throughout the layers of the valve leaflets, even the interstitial cells, but the MMPs showed stronger staining in the ventricularis over the spongiosa. Immortalized endothelial cells increase MMP-2/9 activity by steady shear stress compared to static culture (32) which could be the case here in our *ex vivo* valvular model leading to increased MMP-2/9 activity by shear stress. Since our IHC for cathepsin L and MMP-2/9 was not conclusive as far as specific protein amount, we did not see a difference in cell type expression of these proteases, we used the Western blotting and zymography data as quantitative indicators of protease activity in the total porcine aortic valve exposed to the various mechanical conditions.

The importance of flow characteristics in tissue remodeling and disease progression has been studied by both engineers and pathologists. The aortic valve experiences a complex hemodynamic environment. Three distinct flow regions exist in the aortic valve: the aortic wall of the sinus of valsalva characterized with low, disturbed flow; the side of the leaflets facing the aorta with low shear, laminar flow and the side facing the ventricle with high shear, laminar flow (33).

Implications: The significance of these results is two-fold. First, it helps to improve understanding of the acute responses of the AV to hypertensive pressure.

Recent clinical retrospective studies indicate that the hypertension is a risk factor for valvular disease such as aortic valve stenosis. However, it is not yet clear how hypertensive pressure is involved in this pathophysiology, especially during the early stages of the disease. Results from this study showed that hypertensive pressure could induce changes in valve matrix synthesis within 48 hrs, indicating that valvular matrix remodeling may contribute to the development of aortic valve stenosis in hypertensive patients. The involvement of cathepsins in these processes implicates this protease family as a potential drug target which might slow down the remodeling process induced by abnormal mechanical stimuli. Additionally, these results offer improved strategies for development of tissue engineered heart valves. A tissue engineered heart valve created with biodegradable scaffolds or ECM need to have controlled degradation. Our results suggest that culture under flow can reduce cathepsin mediated proteolysis which could extend the life of the scaffold in the construct.

In summary, this study, for the first time, has shown that cathepsin L, MMP-2, and MMP-9 may be involved in valvular ECM remodeling induced by hypertensive pressure, while cathepsin L may be contributing to remodeling under regions of stagnant flow.

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CHAPTER 5

OSCILLATORY SHEAR STRESS AND LAMINAR SHEAR STRESS DIFFERENTLY REGULATE THE ROLE OF CATHEPSIN K IN VASCULAR REMODELING

Introduction

Cathepsin K, first identified in osteoclasts, (1-4) is known as one of the potent mammalian elastases (4-6) and is capable of cleaving mature, insoluble elastin (4-6) as found in the arterial wall. However, cathepsin K expression and its role in atherosclerosis and abdominal aortic aneurysmal development have not received much attention until recently. Cathepsins K, L, and S, potent elastinolytic proteases, have been identified in atherosclerotic plaques (7, 8) and in neointima following balloon angioplasty (9). In addition, cathepsin activity is increased in abdominal aortic aneurysms (AAA) (7, 10). Smooth muscle cell expression of cathepsin K has been studied, particularly with regard to cytokine induction (8), while endothelial cell expression of cathepsin K is fairly limited to immunostaining in human plaques (11). Lack of cathepsin K in ApoE deficient mice fed a high fat diet reduced the number and size of atherosclerotic lesions, increased collagen content, and decreased breaks in the elastic lamina (11).

The importance of shear stress in vascular biology and pathophysiology has been highlighted by the focal development patterns of atherosclerosis in hemodynamically defined regions. For example, the regions of branched and curved arteries exposed to disturbed flow conditions including oscillatory and low mean shear stresses (OS) correspond to athero-prone areas. In contrast, straight arteries exposed to pulsatile, high levels of laminar shear stress (LS) are relatively well protected from atherosclerotic

plaque development (12). Several human and animal studies have demonstrated that atherosclerotic lesions and aneurisms of the abdominal aorta occur in the regions where they are exposed to unstable flow conditions such as flow reversal, low mean wall shear stress and high oscillatory shear index (13-18).

Our previous findings show that another member, cathepsin L, is inhibited by unidirectional laminar shear stress leading to reduced gelatinase and elastase activity (19). Therefore, we examined the hypothesis that exposure of endothelial cells to OS and LS will regulate cathepsin K expression and activity leading to changes in gelatinase and elastase activity. Our findings show that OS induces cathepsin K expression and activity in comparison to LS, which inhibits this enzyme in endothelial cells suggesting a role for cathepsin K at sites of disturbed flow and involvement in pathological vascular remodeling such as atherosclerosis and AAA.

Methods

Mouse Aortic Endothelial cells (MAEC) culture and shear stress studies. Endothelial cells obtained from the thoracic aortas of C57/BL6 control were isolated and cultured in growth medium growth medium [DMEM containing 20% fetal bovine serum (FBS), 100 μg/ml endothelial cell growth supplement (ECGS, Sigma) and 2.5 U/ml heparin)] as described previously (20) and used between passages 7-10. Confluent endothelial monolayers grown in 100 mm tissue culture dishes were exposed to an arterial level of uni-directional LS (15 dyn/cm^2) or OS with directional changes of flow at 1 Hz cycle (± 5 dyn/cm^2) by rotating a Teflon cone (0.5° cone angle) as described previously by us (21)

One hour prior to shear, the monolayers were washed and changed to 10 ml of fresh shear medium (the growth medium without serum).

Quantitative Real-time PCR. Following shear exposure, cells were washed with ice-cold PBS. Real-time PCR for Cathepsin K was carried out as previously described (22). Briefly, 4 μg of total RNA was reverse-transcribed using random primers and a Superscript-II kit (Invitrogen) to synthesize first-strand cDNA. The cDNA was purified using a microbiospin 30 column (Bio-Rad) in Tris buffer and stored at –20 °C until used. The cDNA was amplified using a LightCycler (Roche Applied Science) RT-PCR machine. The mRNA copy numbers were determined based on standard curves generated with murine Cathepsin K and 18 S templates. The 18 S primers (50 nM at 61 °C annealing temperature; Ambion) were used as an internal control for real-time PCR using a LightCycler and capillaries (Roche Applied Science), recombinant *Taq* polymerase (Invitrogen), and *Taq* start antibody (Clonetech). A quantitative RT-PCR using Cathepsin K primer pair (forward 5'-AAG TGG TTC AGA AGA TGA CGG GAC-3' and reverse, 5'-TCT TCA GAG TCA ATG CCT CCG TTC-3') was carried out using an annealing temperature of 55°C and extension time of 7 s in the PCR buffer (20 mM Tris-Cl, pH 8.4, at 25 °C, 4mM $MgCl_2$ to which was added 250 μg/ml bovine serum albumin, 200 μM deoxynucleotides) containing SYBR green (1:84,000 dilution), 0.05 unit/μl *Taq* DNA polymerase, and Taq Start antibody (1:100 dilution).

Western blots. Following shear exposure, conditioned media were collected and normalized to 10 ml total with fresh serum free shear media if necessary, and

concentrated 20 to 30 fold with a spin concentrator (5 kDa molecular weight cutoff, Vivascience). Cells were rinsed twice with phosphate buffered saline and then lysed with RIPA buffer. Following modified Lowry protein assay, equal amounts of total protein were resolved by SDS-PAGE. Protein was transferred to a polyvinyldifluoride membrane (Millipore), and probed with a mouse monoclonal anti-cathepsin K antibody (1:200, Calbiochem), anti-cathepsin L (1:500, R&D), anti-cathepsin S (1:1000, Santa Cruz), and anti-actin (1:1000, Santa Cruz) and a secondary antibody conjugated to alkaline phosphatase (Bio-Rad), which were detected by a chemiluminescence method (23).

Transwell Assay. MAEC were grown to confluence on gelatin coated Transwell filters with pore size of 0.2 μm in growth medium. Medium was replaced with serum free medium for eight hours. Equal volumes were collected from apical and basal chambers, and proteins were precipitated with ice cold acetone and prepared for Western blotting as described above.

Transfection of siRNA*:* To knockdown mouse cathepsin K mRNAs, the annealed siRNA duplex [sense: 5'-GCA AGC ACU GGA UAA UUA A tt', antisense: 5' UUA AUU AUC CAG UGC UUG C- tt] (MWG Biotech) and nonsilencing duplex [sense: 5'-UUC UCC GAA CGU GUC ACG Utt, antisense: 5'- ACG UGA CAC GUU CGG AGA Att] (Qiagen) were obtained. Sub-confluent (75-80% confluency) MAEC were transfected at a final siRNA duplex concentration of 100 nM using Oligofectamine (Invitrogen) in serum free medium. After 6 hrs, the medium was supplemented with FCS

(final 10% concentration) and cultured an additional 42 hrs prior to exposing the cells to OS, LS, or no flow conditions.

Gelatinase and elastase assay. Five μg/ml of BODIPY® fluorescein-conjugated DQ™ elastin or gelatin (Molecular Probes) in 5 ml of fresh serum-free DMEM was incubated with MAEC following exposure to OS, LS, or St for one day. After an additional 24 hours, aliquots (200 μl) of conditioned media were assayed with a fluorescence plate reader, in triplicate, with background fluorescence subtracted from the no-cell negative control at 485 nm excitation and 525 nm emission.

JPM biotin labeling. Media was collected after 24 hours exposure to OS, LS, or static conditions and centrifugally concentrated with a 5 kDa molecular weight cutoff (Vivascience). Equal aliquots of protein were incubated in buffer containing 50 mM Na Acetate pH 4.2, 1 mM EDTA, 1% Triton, 3 mM DTT, with 20 μM of the biotinylated JPM probe at 37°C for 30 minutes prior to addition of Laemmli buffer, boiling, and separation by a 12.5% SDS-PAGE. Proteins were transferred to PVDF membranes, and probed for biotin with the VectaStain Elite kit (Vector Labs) according to manufacturer's instructions.

Immunohistochemistry*:* Frozen sections of human coronary arteries from transplant patients were acquired and fixed in acetone. After air drying and blocking for 30 minutes with 1% gelatin/PBS, the cathepsin K primary antibody (Calbiochem) was incubated in a 1:20 dilution of 1% BSA/PBS for one hour followed by a PBS rinse then secondary

antibody incubation, conjugated to alkaline phosphatase, diluted in 1% BSA/PBS + 2% normal serum for 30 minutes. Vector ABC kit (Vector) was used for color reaction according to manufacturer's instructions. Hematoxylin was used to counterstain. Negative control was incubated with 1% BSA/PBS in the absence of primary antibody. Slides were viewed with a microscope to visualize color reaction, and then viewed under an inverted fluorescent microscope with FITC filter and laser excitation to view elastin auto-fluorescence.

Statistical Analysis. Student's unpaired t-test was used to establish significance between groups. $P<.05$ was considered statistically significant.

Results

LS decreases cathepsin K mRNA and protein expression in endothelial cells compared to OS.

We initially found that cathepsin K is a mechanosensitive gene by DNA microarray analysis done on MAEC exposed to 24 hours of OS, LS, or no flow (static) conditions and found that OS increases cathepsin K mRNA levels by 2.5 fold over St and LS (Figure 5.1A). To verify this finding, we employed quantitative real time PCR assay using 18S as an internal control. MAEC exposed to OS showed a 2.4- and 1.7-fold higher cathepsin K mRNA levels than those exposed to static and LS, respectively (Figure 5.1B). Cathepsin K mRNA levels between static and LS were not significantly different.

Steady laminar shear stress also significantly decreased pro- and mature cathepsin K protein levels in the cell lysate by 55% and 80%, respectively, after 24 hours exposure to OS, LS, or St control (Figure 5.1C). However, MAEC cultured under no flow conditions contained more twice as much cathepsin K as OS stimulated cells.

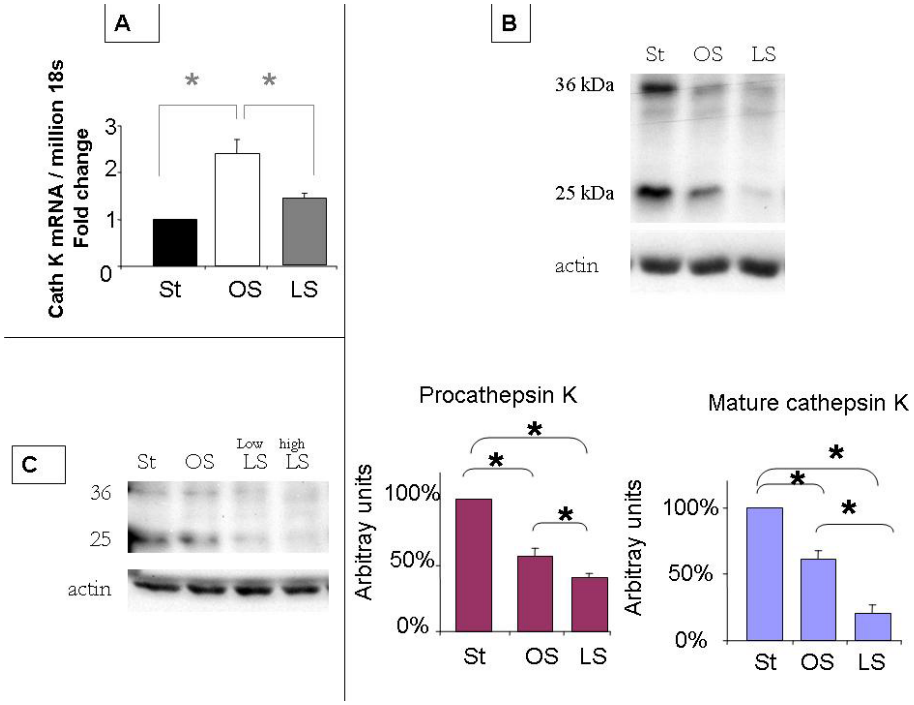

Figure 5.1. OS and LS differentially regulate cathepsin K mRNA and protein expression in endothelial cells. Confluent MAEC were exposed to Static (St), oscillatory shear (OS), or laminar shear stress (LS) for 1 day. Following shear, total RNA from cell lysates for real time PCR were prepared or cells were lysed for Western blot. The bar graph in Panel A shows *cathepsin K* mRNA levels normalized against the 18S and expressed as fold change of static control values (mean ± SEM, * $p<0.05$, n= 5 to 7). Panel B shows a representative Western blot using an equal amount of protein from cell lysates and probed with an antibody to cathepsin L and a β actin antibody as an internal control. Densitometric quantification is shown in the bar graphs (mean ± SEM, n=4, *$p< .05$). Panel C is a representative blot from 2 independent experiments using MAEC exposed to static culture, ± 5 dyn/cm^2 OS, 5 dyn/cm^2 LS, and 15 dyn/cm^2 LS.

Low and oscillatory shear stresses are found at sites of disturbed flow leading to atherosclerosis and AAA development, so to determine whether it was the direction or

the magnitude of the shear stress that was regulating cathepsin K protein levels, we exposed MAEC to lower levels (5 dynes/cm^2) of steady laminar shear stress equal in magnitude to the OS profile, but in one direction. From figure 5.1C, the lower magnitude of LS still inhibits MAEC expression of cathepsin K by 35% for the proform and 74% for the mature form illustrating the importance of unidirectional flow suppressing cathepsin expression. Furthermore, higher magnitude of shear stress reduced the level even more (49% for proform and 82% for the mature form) suggesting that the magnitude of shear stress is inversely related to cathepsin K expression in MAEC.

MAEC secrete cathepsin K in a shear regulated manner and preferentially in the basal direction.

Since cathepsin K is a secreted protein in osteoclasts (4), we examined the protein levels in the conditioned media of MAEC exposed to OS, LS and no flow conditions for 2 to 24 hrs. The pro-cathepsin K protein (37 kDa) secreted into the conditioned media in a time-dependent manner was readily detected in cells exposed to static and OS groups (Figure 5.2A). However, pro-cathepsin K protein level was extremely low in the conditioned media of LS exposed cells. After 1 day of LS exposure, pro-cathepsin K level in LS group was 21% of OS group (Figure 2A). Interestingly, the mature cathepsin K form (25 kDa) was detectable only in MAEC exposed to OS, but not in those of static and LS (Figure 5.2A).

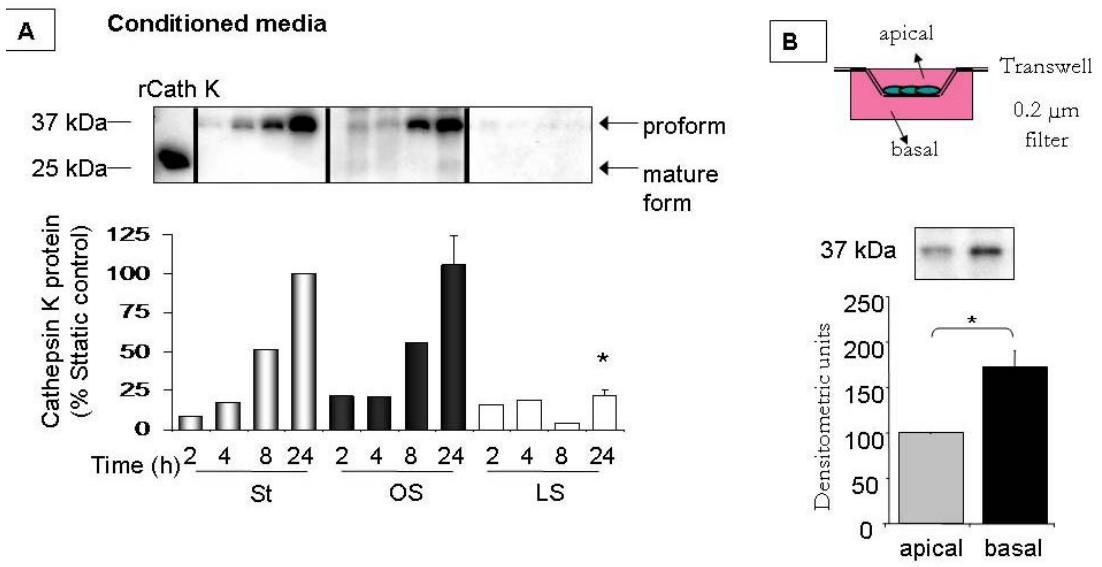

Figure 5.2. MAEC secrete cathepsin K in a shear regulated manner and preferentially in the basal direction. A) Confluent MAEC were exposed to Static (St), oscillatory shear (OS), or laminar shear stress (LS) for 2 to 24 hr. Recombinant cathepsin K (rCath K) was used as a positive control. The procathepsin K band (37 kDa) was densitometrically quantified and presented as % of 24 hr static control as shown in the bar graph (mean ± SEM, * $p<0.05$, n= 2 to 5). Note that only MAEC exposed to OS show a mature, active cathepsin K band (25 kDa) in their conditioned media. B) MAEC were grown to confluence on Transwell filter with 0.2 μm pore size and media was sampled from the apical and basal chambers, then equal aliquots of protein were used for Western blot (mean ± SEM, * $p<0.05$, n= 5).

Endothelial cells line the blood vessel wall and are capable of secreting proteins apically, into the bloodstream, or basally, into the vessel wall. For cathepsin K to function to remodel the vessel wall, we hypothesized that endothelial cells secrete it in the basal direction. We tested this hypothesis using a Transwell filter assay. MAECs were grown to confluence on the 0.2 μm filter to allow for proteins to pass, but not cells. After an 8 hour incubation in serum free medium, the conditioned medium was collected and assayed for cathepsin K protein. It was clear that MAEC preferentially secrete procathepsin K in the basal direction, although there is apical release of the protein (Figure 5.2B). These MAEC were cultured under static conditions in which mature

cathepsin K is not detected by Western blot. We verified that cathepsin K was not moving through the endothelial cell layer by addition of mature cathepsin K to the apical or basal chamber, but it was not detected in the opposite chamber after the period of incubation (data not shown).

Cathepsin K siRNA knocks down cathepsin K protein and activity.

We decided to use a siRNA approach to determine the specific contribution of cathepsin K to extracellular matrix proteolytic activity which we have previously shown to be increased by OS and to be decreased by LS and to be cathepsin dependent (19), but it was important to first validate the specificity of the siRNA towards cathepsin K over cathepsins L and S, the other highly elastinolytic and collagenolytic family members. Treatment of MAEC with cathepsin K siRNA significantly knocked down cathepsin K protein expression as shown in Western blots of the cell lysates (Figure 5.3A) and the conditioned media (Figure 5.3B). In contrast, a non-silencing siRNA control had no effect on the protein levels. Transfection of the siRNA does not affect the reduction in cathepsin K protein by LS. It is also evident that cathepsin S is downregulated by LS in a similar fashion to cathepsin K, both intracellularly and extracellularly.

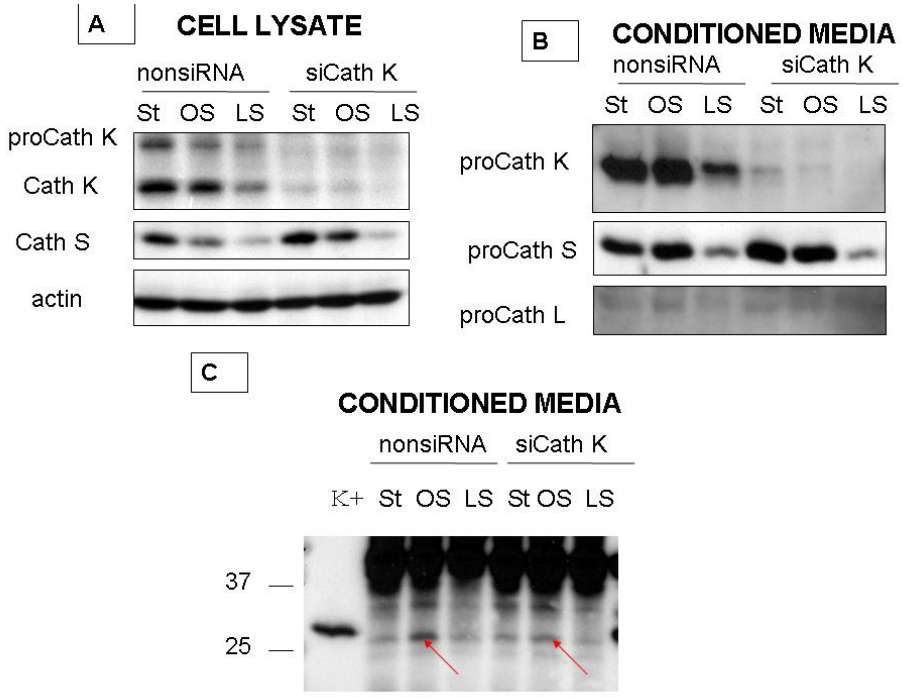

Figure 5.3. Cathepsin K siRNA knocks down cathepsin K protein and activity. Sub-confluent MAECs were transfected with cathepsin K specific siRNA (100 nM) or nonsilencing RNA (non siRNA) 24 hours prior to shear exposure. Transfected cells were then exposed to OS, LS, or static conditions for 1 day. Cathepsin K protein knockdown by cathepsin K siRNA was confirmed by Western blot using cell lysates (A) and conditioned media (B). Samples were probed with antibodies to cathepsins K, L, and S, and a β actin antibody as an internal control.

By Western blot, procathepsin K was highly detected while the mature form was faintly detected under OS (Figure 5.2A); the mature form is responsible for proteolytic activity. We employed the use of the biotinylated, activity based probe JPM to detect any active, mature cathepsin K secreted into the conditioned media in response to OS. The mature cathepsin K band migrates similarly to other cathepsin family members in this assay, and since the biotinylated probe detects many active cathepsins, we also transfected cells with cathepsin K siRNA to compare which bands contained the cathepsin K signal. Recombinant cathepsin K labeled with the biotinylated JPM was also used as a positive control and as an aid in locating mature cathepsin K secreted by the

MAEC in response to shear. Figure 5.3C shows that there is an increase in the band near the cathepsin K positive control in response to OS which confirms our findings from figure 5.2A. Confirmation that this is the cathepsin K band is seen in the reduction of its intensity in the conditioned media collected from MAEC transfected with cathepsin K siRNA suggesting that OS does increase the level of mature, active cathepsin K in the extracellular environment where it may be capable of degrading the extracellular matrix comprising the blood vessel wall.

Cathepsin K contributes to endothelial cell elastase activity, and Cathepsin K siRNA knocks down shear mediated gelatinase activity.

From the above result, it was clear that MAEC were producing active cathepsin K in response to OS, and we had previously shown that OS increases elastase and gelatinase activity of MAEC compared to LS, with cathepsins contributing greatly. Taken together, we hypothesized that cathepsin K contributed to this extracellular matrix proteolytic activity, the extent of which was unknown. To test this hypothesis and determine cathepsin K's involvement in this shear regulated activity, cathepsin K siRNA treatment of MAEC was used in conjunction with the fluorescent elastase and gelatinase assays. Cathepsin K knockdown with siRNA reduced MAEC elastase activity by 30% in response to OS and St conditions (Figure 5.4A). As expected from previous findings, LS significantly reduces the elastase activity in the nonsilencing controls compared to OS and St, but interestingly, there was no further reduction when cathepsin K was knocked down, presumably because LS reduced MAEC expression of cathepsin K leaving only a small amount of protein to knockdown further.

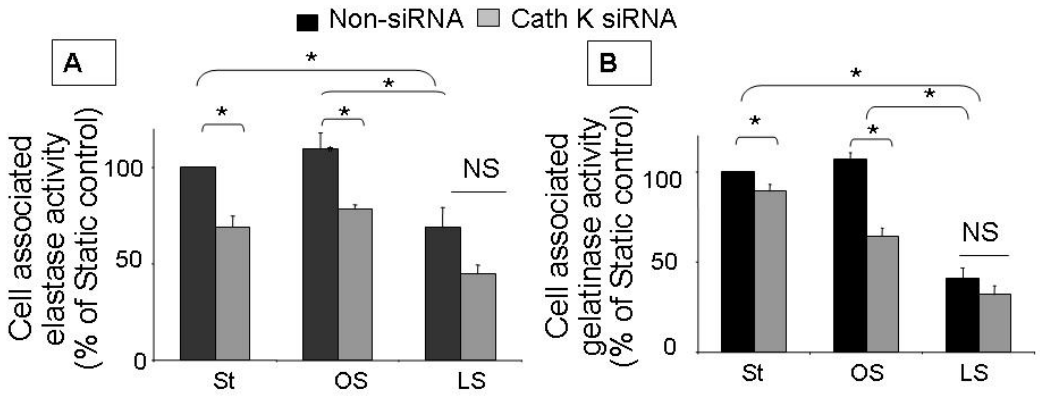

Figure 5.4. Cathepsin K contributes to endothelial cell elastase activity, and Cathepsin K siRNA knocks down shear mediated gelatinase activity. MAEC were transfected with cathepsin K specific siRNA (100 nM) or non-silencing RNA (non siRNA) control 48 hours prior to shear. Transfected cells were then exposed to OS, LS, or static conditions for 1 day. Following shear, BODIPY -gelatin or -elastin were added to intact cells and incubated overnight in fresh media. Elastase (**A**) and gelatinase (**B**) activities were measured as an increase in fluorescence intensity. The bar graphs show % of static control values (mean ± SEM, * $p<0.05$, n= 3 to 4).

Cathepsin K knockdown affected gelatinase activity slightly differently. LS reduced this activity by almost 50% in the nonsilencing controls, and again, there was no further reduction of gelatinase activity after knocking down cathepsin K (Figure 5.4B). This again confirms that LS inhibits cathepsin K to such low levels in MAEC that further knockdown does not produce significant changes. It does significantly reduce gelatinase activity of MAEC exposed to OS by 43% and of Static by a small but significant 11%.

Endothelium overlying disrupted IEL expresses cathepsin K. To provide physiological evidence for the role of cathepsin K in arterial remodeling, we performed tissue staining of minimally diseased coronary arteries vs. diseased arteries using an anti-cathepsin K antibody. We found stronger staining of cathepsin K over disrupted IEL

using elastin auto fluorescence. This double blind grading of endothelial cell cathepsin K staining intensity and disrupted IEL had a correlation of .74 (Figure 5.5).

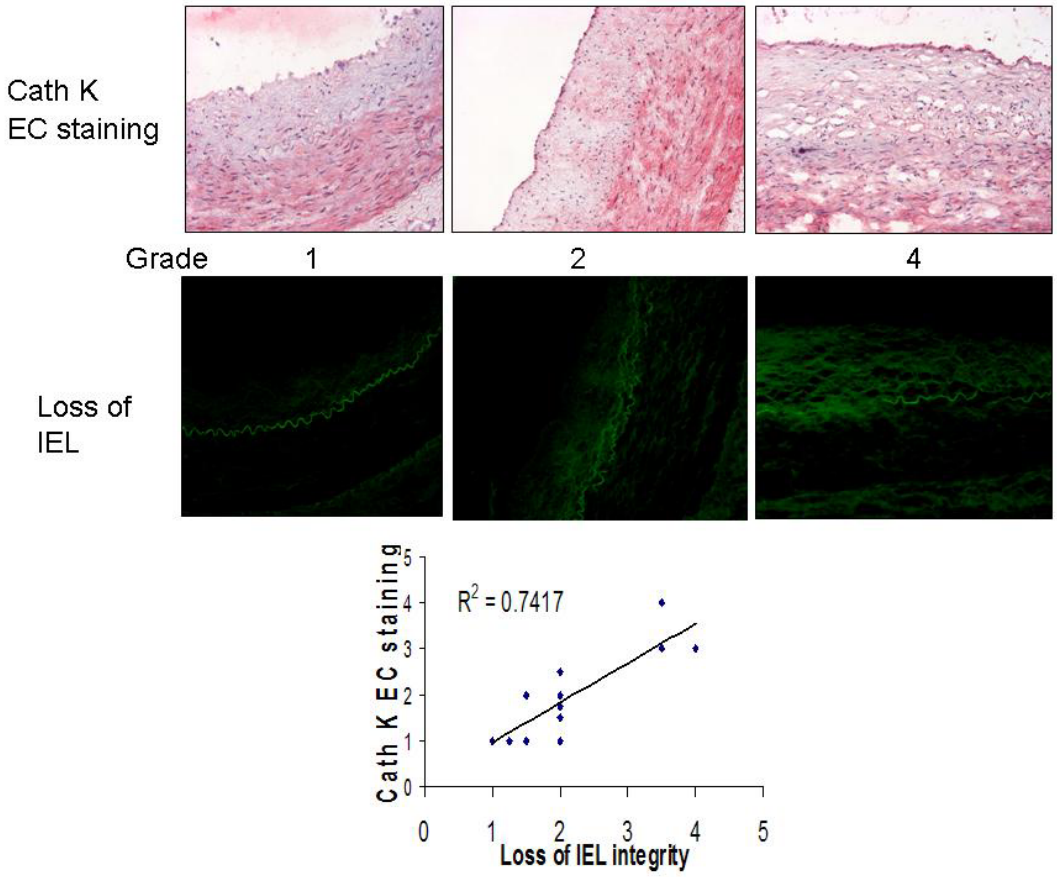

Figure 5.5. Endothelium overlying disrupted IEL expresses cathepsin K. Immunohistochemical staining of human coronary arteries for cathepsin K in minimally diseased (A) and atherosclerotic (B) regions. Autofluorescence of elastic laminae reveals breaks in the structures. (C) Double blind grading of endothelial cell staining intensity and internal elastic laminar loss of integrity.

Discussion

The novel findings of this study are that (1) OS increases endothelial cell cathepsin K mRNA and protein levels as well as activity over that of LS and (2) OS increases endothelial cell secretion of mature cathepsin K over St and LS conditions (3) endothelial cells show a basal preference for secretion of cathepsin K (4) shear stress

regulation of cathepsin K activity moderates endothelial cell gelatinase and elastase activity; and (5) endothelial cells in human coronary arteries overlying disrupted IEL express cathepsin K. These results support our earlier finding that another member of the cathepsin family is regulated by shear stress.

Here, we showed that OS upregulates cathepsin K mRNA level by 2.4 fold over static control (Figure 5.1A) but there is more protein in the MAEC cultured under static conditions (Figure 5.1B) which suggests that there could be some post-transcriptional regulation of cathepsin K mRNA. However, it is also likely that since cathepsin K is activated by OS (as shown in Figure 5.2A and 5.3C) then the active protease could be capable of degrading itself leading to a lower steady state level of the enzyme in the cell; other cathepsins are activated by OS as well (19) and are responsible for protein turnover inside of the cell. Cathepsin K has been shown to also autolytically activate itself by cleaving its own propeptide (24). Once active, mature cathepsin K is present, then those molecules are capable of cleaving the propeptides off of others in solution (25).

Figure 5.2A shows that mature cathepsin K (25 kDa) is detectable by Western blot extracellularly only from those cells that were exposed to OS which seems to account for the greater amount of active cathepsin K detected with the activity based probe in figure 5.3C. It was interesting to note that mature and active form of cathepsin K was observed only in media from OS exposed cells, while the level of pro-cathepsin K was similar between the static and OS group. This suggests that OS may be involved in stimulating conversion of the pro-form to the mature form, but the underlying mechanisms are not clear. Knockdown of cathepsin K with siRNA significantly inhibited gelatinase and elastase activity of the static and OS treated cells, but not that of the LS

group (Figure 5.4A, B). Once again, we refer back to the activation of cathepsin K by OS (Figure 5.2A, 5.3C) and the greatest reduction in matrix proteolytic activity seen with gelatinase activity (Figure 5.4A). Cathepsin K is a powerful collagenase, capable of cleaving triple helical Type I collagen both at the telopeptides and intra-helically, a characteristic unique to this enzyme (6). Since gelatin is denatured collagen, we accept that cathepsin K may have more of an effect on the turnover of that substrate. Li et al have reported that binding of glycosaminoglycans (GAGs) regulate cathepsin K activity towards type I collagen; chondroitin sulfate increases cathepsin K collagenolytic activity by forming multimeric complexes while dermatan sulfate, heparin sulfate, and heparin all inhibit this activity and compete for the active site of cathepsin K (26). Elastase activity was not examined so it is unknown at present if it is altered by GAG binding. These alternative factors that regulate cathepsin K extracellular matrix proteolytic activity could serve as additional protectants against uncontrolled degradation after release of cathepsin K in disease conditions, such as oscillatory shear stress and atherosclerosis. An investigation of how shear stress regulates endothelial cell GAG production could provide some insight into these regulations of cathepsin K activity.

Endothelial cells are polarized cells with an apical surface exposed to the blood flowing through the lumen of the vessel and a basal portion that sits on basement membrane and the other layers of the vessel wall. This polarity has been shown to be important in different experiments including a previous study in our lab showing how caveolae form on the apical side in response to shear stress (27). The current study‘s report that cathepsin K is secreted preferentially out of the basal surface of the cell gives a context for its secretion into the vessel wall and suggests that shear stress can regulate

cathepsin K presence and activity in vascular remodeling. Cathepsin K has been co-localized with endothelial cells in human atherosclerotic samples as well as with smooth muscle cells (SMCs) and macrophages (11). Endothelial cell contribution of cathepsin K may work mainly in the initial phases of atherosclerosis when the IEL is first degraded to signal SMC migration into the subintimal space, and it could be important in later stages of the plaque leading to plaque rupture. In fact, transient expression of cathepsin K in human plaques has been demonstrated (11). Many plaques rupture at the shoulder regions for a mechanical and biological reasons (28, 29), and the endothelium is still present at these areas. Also, fibrous plaques are composed mainly of collagen, a prime substrate for cathepsin K hydrolysis. Local secretion of cathepsin K in response to OS at these sites could be the metaphoric "straw" that breaks the plaque. Even procathepsin K secreted into the subintimal space can be activated as macrophages in the vessel wall locally acidify the environment after coming into contact with elastin (30) and cathepsin K can autolytically activate itself under acidic conditions (25). Atherosclerotic plaque environments also display pH heterogeneity with lower pH found near large lipid pools, presumably because of the greater presence of lipid-laden macrophages (31). Evidence of cathepsin K at the site of plaque rupture has already been demonstrated in human samples, and the absence of cathepsin K in mice increased plaque collagen content but reduced plaque progression(11).

Our result of tissue staining of cathepsin K in the endothelium of human coronary arteries increasing with damage to the IEL (Figure 5.5) opens the possibility of using endothelial cell cathepsin K clinically as a biomarker for atherosclerosis. Transient expression of cathepsin K at different stages of the atherosclerotic development has been

shown in humans (11); biomarkers for endothelial cell cathepsin K could be used as predictors of sites of plaque rupture. The tissue staining correlation also supports our hypothesis that this enzyme works to breakdown structures of the arterial wall during plaque formation. Elastases span different classes of proteases that must be capable of hydrolyzing mature, insoluble elastin. Serine proteases, MMPs, and cysteine proteases all have members that are elastinolytic, but the serine proteases and MMPs have been the focus of attention as arterial elastinolytic enzymes. Recent studies, however, have shown the important role of cathepsins and their inhibitor cystatin C in elastin degradation and atherosclerosis development. Sukhova et al (32, 33) demonstrated that mice deficient in cathepsin S in LDL receptor-null mice show decreased internal elastic lamina fragmentation and reduction in atherosclerosis, while mice deficient for the inhibitor, cystatin C, had increased IEL fragmentation. With respect to this, we have previously showed that cathepsin L gelatinase and elastase activity is also regulated by shear stress in endothelial cells (19), however, this current study is the first to show that shear stress regulates cathepsin K in endothelial cells as well.

Arteries respond to changes in blood flow to maintain healthy levels of wall shear stress, and this process involves more than vasodilation and vasoconstriction, but it also includes the actual degradation and remodeling of the structures of the vessel wall in an endothelial cell-dependent manner. Low blood flow conditions following angioplasty causes decreased lumen size due to inward remodeling of the vessel (34), while large increases in blood flow can increase the outward remodeling of the vessel (35). Flow dependent arterial remodeling has been shown to be endothelium-dependent (36). Increases in blood flow during development of the vasculature or after surgically creating

an arterio-venous fistula cause remodeling of the internal elastic lamina (37). Taken together with the current findings, we suggest that mechanosensitive regulation of cathepsins and elastase and gelatinase activities play a role in the flow-dependent vascular remodeling.

Our study provides insight into a potential mechanism by which the vascular structures and the internal elastic lamina can be degraded under disturbed flow conditions. Based on our findings, we propose that OS increases cathepsin K expression and activity in endothelial cells, which, in turn stimulates gelatinase and elastase activity. The increased elastase activity then degrades elastins in the internal elastic lamina, resulting in their fragmentation, subsequent arterial wall remodeling, and atherosclerotic plaque development. At later stages, this increased activity could contribute to fibrotic plaque rupture due to the powerful collagenase activity of cathepsin K. In summary, we showed that cathepsin K is a mechanosensitive enzyme, which has a potential importance in vascular remodeling and atherosclerosis.

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CHAPTER 6

CATHEPSIN LOCATIONS IN ENDOTHELIAL CELLS

Introduction

Cathepsins, traditionally referred to as the lysosomal cysteine proteases, are being discovered as having functions outside of these compartments. Cathepsins S, K, and L are secreted by various cell types including macrophages (1-4), osteoclasts (5, 6), chondrocytes (7), and others. Membrane bound cathepsin B is involved in angiogenesis (8) and tumor metastasis (9-11). Cathepsin S binds to integrin alpha V beta 3 on vascular smooth muscle cells in response to cytokine stimulation (12). Upon overexpression in tumor cells, cathepsin L aggregates in multivesicular bodies near the cell surface (13).

Together, these support the concept that cathepsins may not be just in lysosomes in endothelial cells or that their location may change in response to shear stress. Caveolin-1 moves from the Golgi apparatus to the plasma membrane in response to LS (14), and several G proteins also change cellular location after induction of LS (15, 16).

Since our findings are that oscillatory shear stress (OS) increases cathepsin K and S protein level and cathepsin L activity in mouse aortic endothelial cells (MAEC), we investigated the hypothesis that shear stress may regulate the intracellular locations of cathepsins using immunocytochemistry and cell fractionation.

Methods

Mouse Aortic Endothelial cells (MAEC) culture and shear stress studies. Endothelial cells obtained from the thoracic aortas of C57/BL6 control were isolated and cultured in growth medium growth medium [DMEM containing 20% fetal bovine serum (FBS), 100

μg/ml endothelial cell growth supplement (ECGS, Sigma) and 2.5 U/ml heparin)] as described previously (17) and used between passages 7-10. Confluent endothelial monolayers grown in 100 mm tissue culture dishes were exposed to an arterial level of uni-directional LS (15 dyn/cm^2) or OS with directional changes of flow at 1 Hz cycle (± 5 dyn/cm^2) by rotating a Teflon cone (0.5° cone angle) as described previously by us (18) One hour prior to shear, the monolayers were washed and changed to 10 ml of fresh shear medium (the growth medium without serum).

Western blots. Following shear exposure, conditioned media were collected and normalized to 10 ml total with fresh serum free shear media if necessary, and concentrated 20 to 30 fold with a spin concentrator (5 kDa molecular weight cutoff, Vivascience). Cells were rinsed twice with phosphate buffered saline and then lysed with RIPA buffer. Following modified Lowry protein assay, equal amounts of total protein were resolved by SDS-PAGE. Protein was transferred to a polyvinyldifluoride membrane (Millipore), and probed with a mouse monoclonal anti-cathepsin K antibody (1:200, Calbiochem), anti-eNOS (1:1000, Cell Signaling), and annexin II (1:1000, Santa Cruz) and a secondary antibody conjugated to alkaline phosphatase (Bio-Rad), which were detected by a chemiluminescence method (19).

Immunocytochemistry. Cells were rinsed with PBS, and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS, and the fixative was quenched with 50 mM ammonium chloride. Blocking buffer of 3% BSA in PBS was preincubated with the cells for an

hour, followed by overnight incubation with the primary antibody: anti-cathepsin L (1:100, Santa Cruz), anti-cathepsin S (1:100, Santa Cruz), anti-cathepsin K (1:50, Calbiochem), anti-GM 130 (1:100), anti-caveolin-1 (1:100), and rhodamine phalloidin (1:100). Alexa 488 conjugated secondary antibodies were used to fluorescently label the primary antibody. Cells were rinsed with PBS then covered with Prolong anti-Fade Kit (Molecular Probes) and visualized on an epifluorescent microscope.

Subcellular proteome extraction. After shear exposure, cytosol was extracted with 10 mM PIPES, 5 mM EDTA, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.01% (w/v) digitonin for 10 minutes. Membrane/organelles were extracted with 10 mM PIPES, 3 mM EDTA, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) Triton X-100 for 30 minutes. Nucleus was extracted in 10 mM PIPES, 0.5% deoxycholate, pH 7.4, 1 mM $MgCl_2$, 1% (v/v) Tween-40, 10 mM NaCl for 10 minutes. After the stage of extraction, the plate was fixed with 4% paraformaldehyde and immunolabeled according to immunocytochemistry protocol described above.

Plasma membrane cell fractionation. Six plates were used for each shear condition. Cells were washed twice with ice-cold PBS. Then they were washed twice with 5 ml buffer A (0.25 M sucrose, 1 mM EDTA, 20 mM Tricene, pH 7.8) and scraped in 3 ml buffer A. Whole cell lysate aliquot was collected here. Cells were pelleted by centrifugation for 5 minutes at 2500 x g and resuspended in 1 ml buffer A. Cells were homogenized with a Wheaton tissue grinder with 20 strokes on ice. Homogenate was transferred to a microfuge tube and centrifuged at 100 x g for 20 minutes to pellet

nucleus. Post nuclear supernatant (PNS) was removed and stored on ice with re-homogenization of the pellet and collection of PNS. The PNS was layered over 30% Percoll in buffer A and centrifuged at 84,000 x g for 30 minutes. The plasma membrane fraction was the visible band in the middle of the ultracentrifuge tube and was collected with a syringe.

Versene wash. Cells were washed with 0.53 mM EDTA in PBS to collect any proteins bound in a calcium dependent manner. Proteins in the supernatant were precipitated in ice cold acetone to prepare for Western blot.

Magic Red^{TM} substrate. Assay was performed according to manufacturer's instructions (Immunochemistry Technologies, LLC). Briefly, the bi-substituted fluorophore cresyl violet is linked to two leucine-arginine $(Z\text{-}LR)_2$ motifs rendering it nonfluorescent and susceptible to cathepsin K cleavage after which red fluorescence accumulates in the cell's organelles. Acridine orange (0.1% v/v) was used to label lysosomes and acidic organelles, and Hoechst was used for nuclear labeling (0.5% v/v). Live cells were viewed under fluorescent microscope with the rhodamine filter for cresyl violet and acridine orange and the UV filter for Hoechst.

Results

Cathepsin L and S are in lysosomes.

Immunostaining of MAEC exposed to OS, LS, or no flow control with cathepsin K, L, and S antibodies was done to find their intracellular location. Cathepsins L and S

both showed a more lysosomal staining pattern as expected since these are the lysosomal family of cysteine proteases (Figure 6.1-6.2). Capturing images under the same settings on the confocal microscope shows that there is a reduced signal in MAEC exposed to LS which supports our central hypothesis that LS inhibits endothelial cell production of cathepsins.

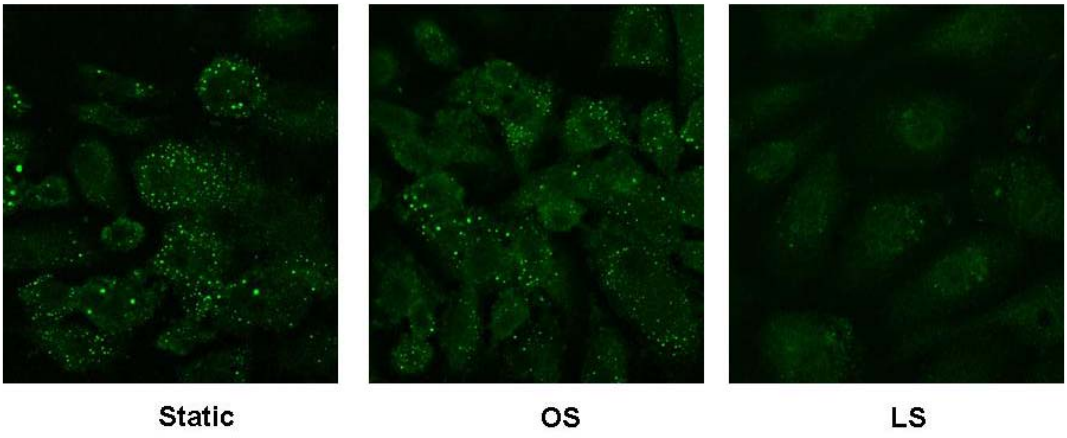

Figure 6.1. Cathepsin L immunocytochemistry.

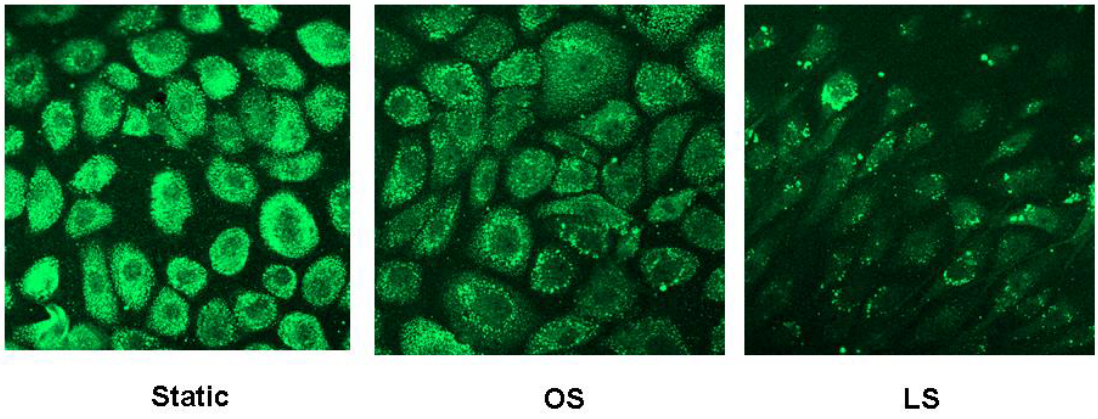

Figure 6.2. Cathepsin S immunocytochemistry.

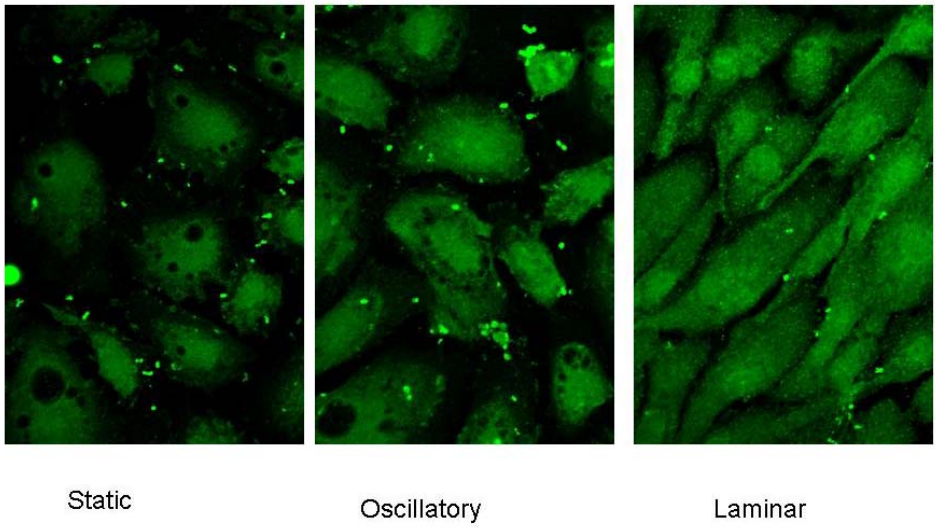

Figure 6.3. Cathepsin K immunocytochemistry.

Cathepsin K positive labeling is in cytosol, nucleus, and cell membrane structures.

Confluent MAEC were exposed to 24 hours OS, LS, or St and then fixed for immunostaining. Cathepsin K was positive in the cytoplasm and in structures that were on the cell periphery with more found in MAEC exposed to OS (Figure 6.3). Preincubating the antibody with the blocking peptide failed to detect cathepsin K in these structures and in the cytoplasm showing that this is not a nonspecific signal (Figure 6.4). These structures appeared to be vesicular in nature which could have been a mechanism by which cathepsin K was being secreted by MAEC. A Z-series of the endothelial cells revealed a hollow center in the structure (Figure 6.5). Cathepsin K is known to be lysosomal, and our use of the Magic Red substrate, $(Z\text{-}LR)_2$, that enters the cell and fluoresces when cleaved by cathepsin K, shows lysosomal activity staining that matches with acridine orange, a molecule that localizes to acidic compartments such as lysosomes (Figure 6.6).

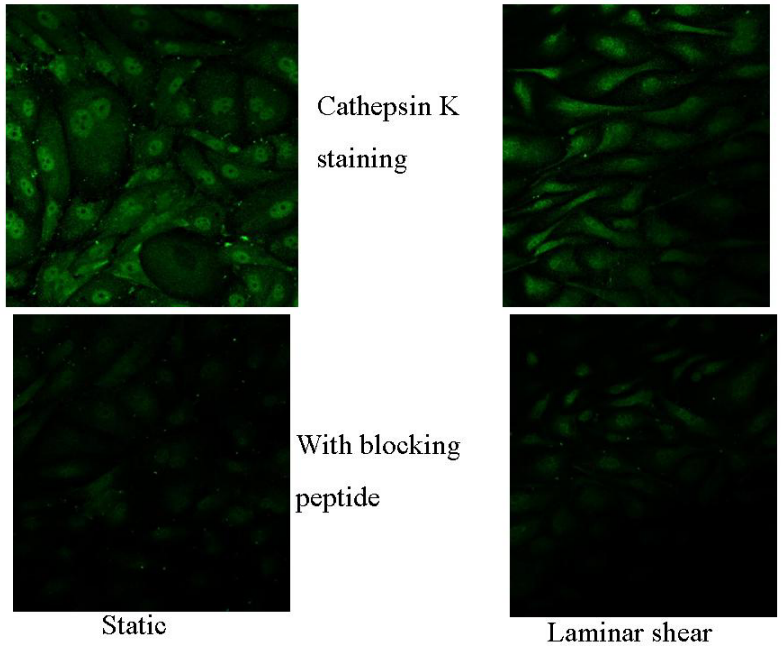

Figure 6.4. Cathepsin K localizes to cell surface on membrane structures. MAECs were exposed to static flow or LS, fixed, and incubated with anti-cathepsin K antibody (upper panels) or blocked anti-cathepsin K antibody blocked with the antigenic peptide (lower panels).

Next, a subcellular proteome extraction was performed to stepwise remove layers/organelles from the cell to determine more specifically where cathepsin K was. The staining pattern changed at each step of the increased detergent strength. After removing the cytosol and any loosely bound membrane structures with digitonin, the structures at the cell periphery were no longer visible (Figure 6.7). Also, instead of the diffuse cytosolic staining seen before, cathepsin K could be seen in distinct vesicles inside of the EC in a pattern similar to lysosomes; again blocked antibody revealed that this staining was specific for cathepsin K. Further detergent to remove the plasma membrane and organelles leaving cytoskeleton and lipid raft components still stain positively for cathepsin K, particular at the cell periphery and in the nucleus (Figure 6.7B). Removal of the nucleus at the next step still shows cathepsin K present with the cytoskeletal and lipid rafts of the cell (Figure 6.7C).

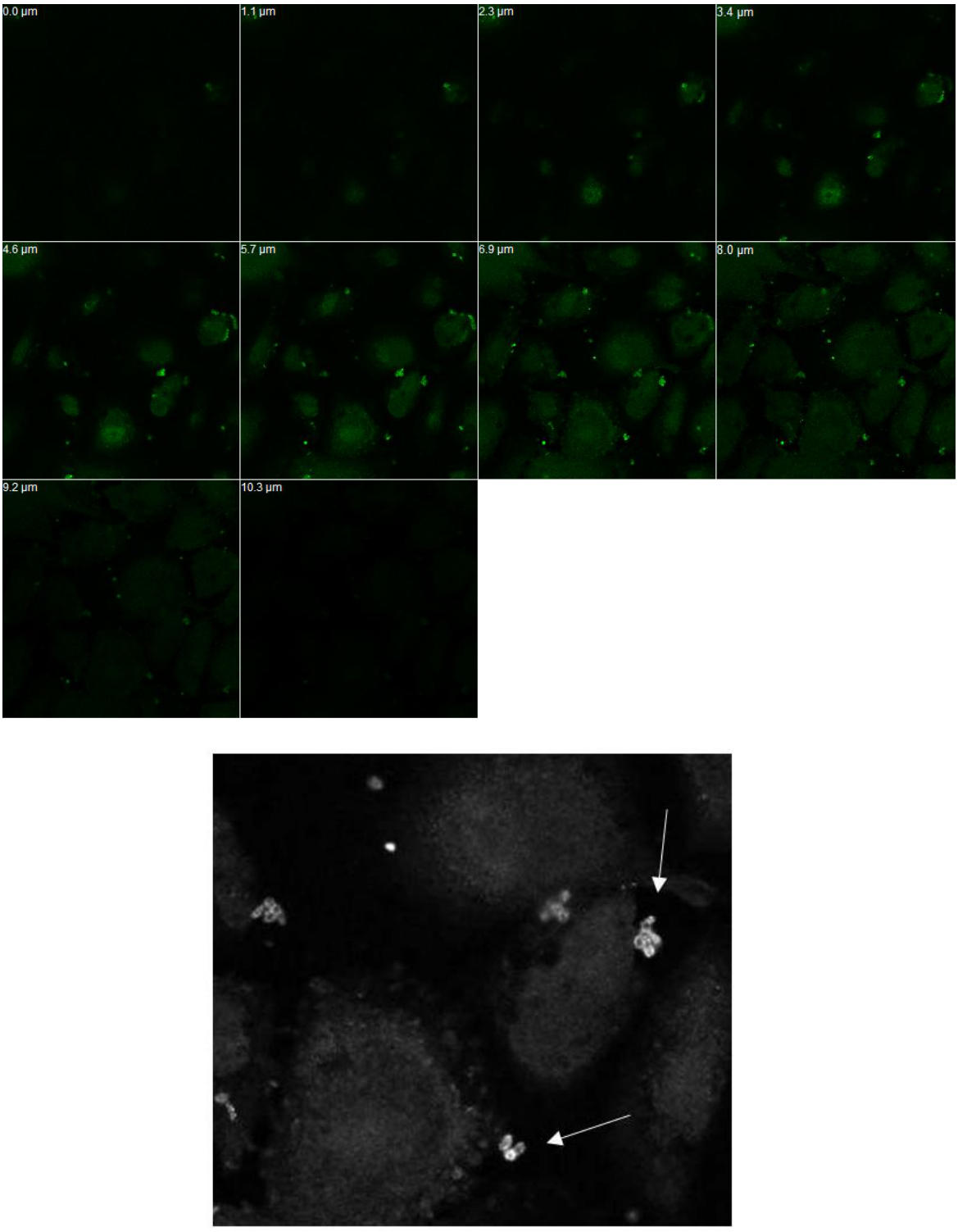

Figure 6.5. Cathepsin K localizes to hollow vesicular like structures on cell periphery. Z-series captured with confocal microscope after immunolabeling with anti-cathepsin K antibody. Close-up of hollow vesicular structures.

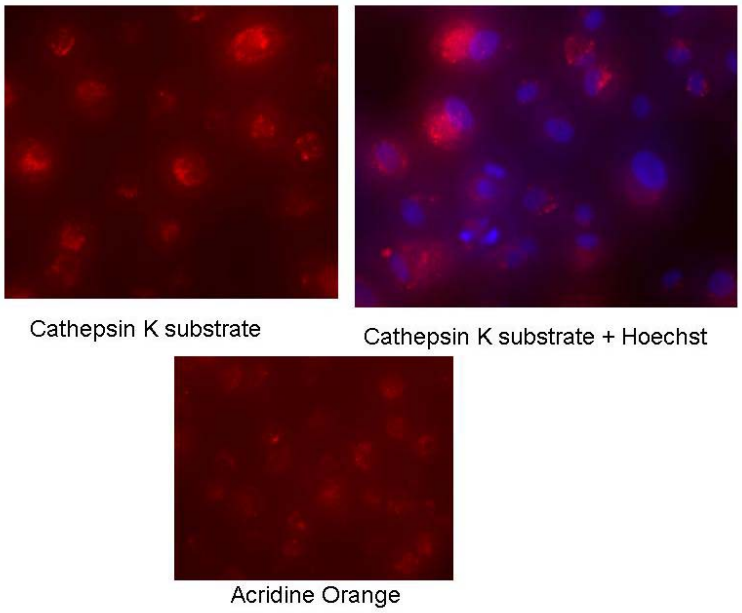

Figure 6.6. Active cathepsin K intracellularly shows lysosomal staining pattern. Cell permeable cathepsin K substrate Z-$(LR)_2$ was incubated with static cultured MAEC to visualize cathepsin K activity. Acridine orange was used to label lysosomes, and Hoechst labeled cell nuclei.

To show a specific co-localization of cathepsin K with known proteins that reside in these different cellular structures, we double labeled the cell caveolin-1, the central protein that comprises caveolae, specialized lipid raft structure, and with phalloidin to see filamentous actin. There appeared to be some co-localization of cathepsin K with caveolin-1 as seen by the faint yellow staining after overlaying the separate images (Figure 6.8). There was yellow staining between cathepsin K and actin suggesting that cathepsin K is associating with cytoskeletal structures under OS, LS, and St conditions (Figure 6.9). Both of these structures are present on the cell membrane.

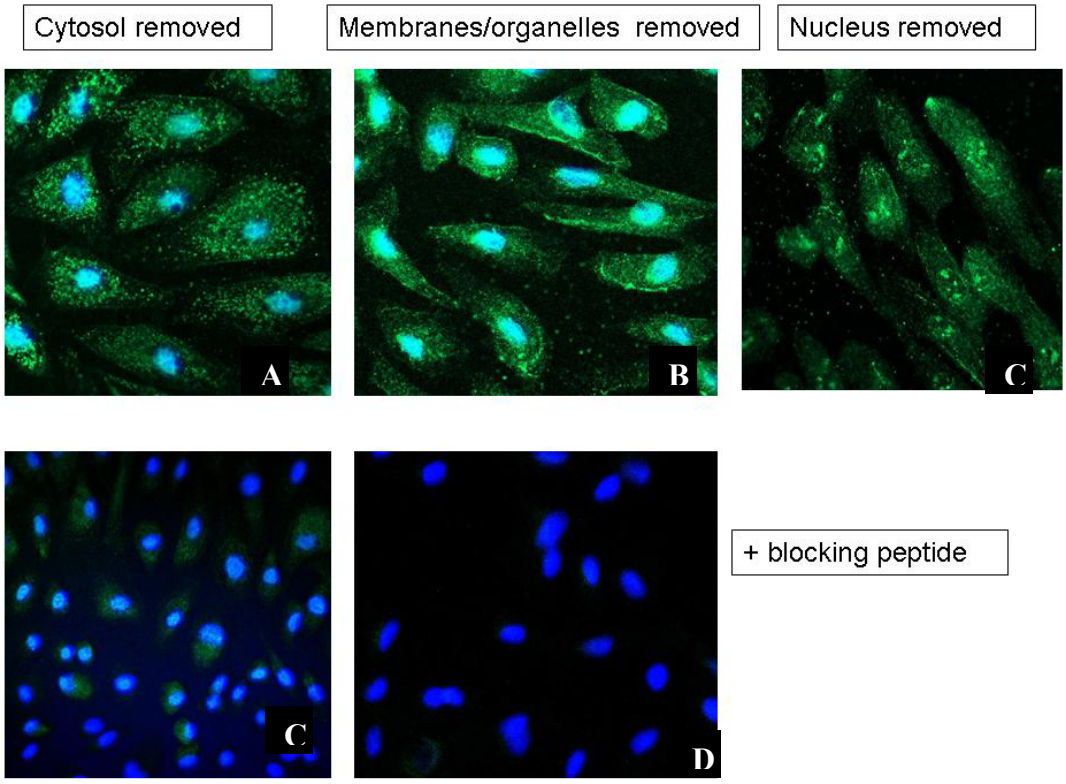

Figure 6.7. Subcellular proteome extraction reveals different cathepsin K locations in the endothelial cell. Cathepsin K staining with the antibody or blocked antibody reveal its location in MAEC. Disappearance of the green staining after incubation with the antigenic peptide illustrates the specificity of the staining for cathepsin K.

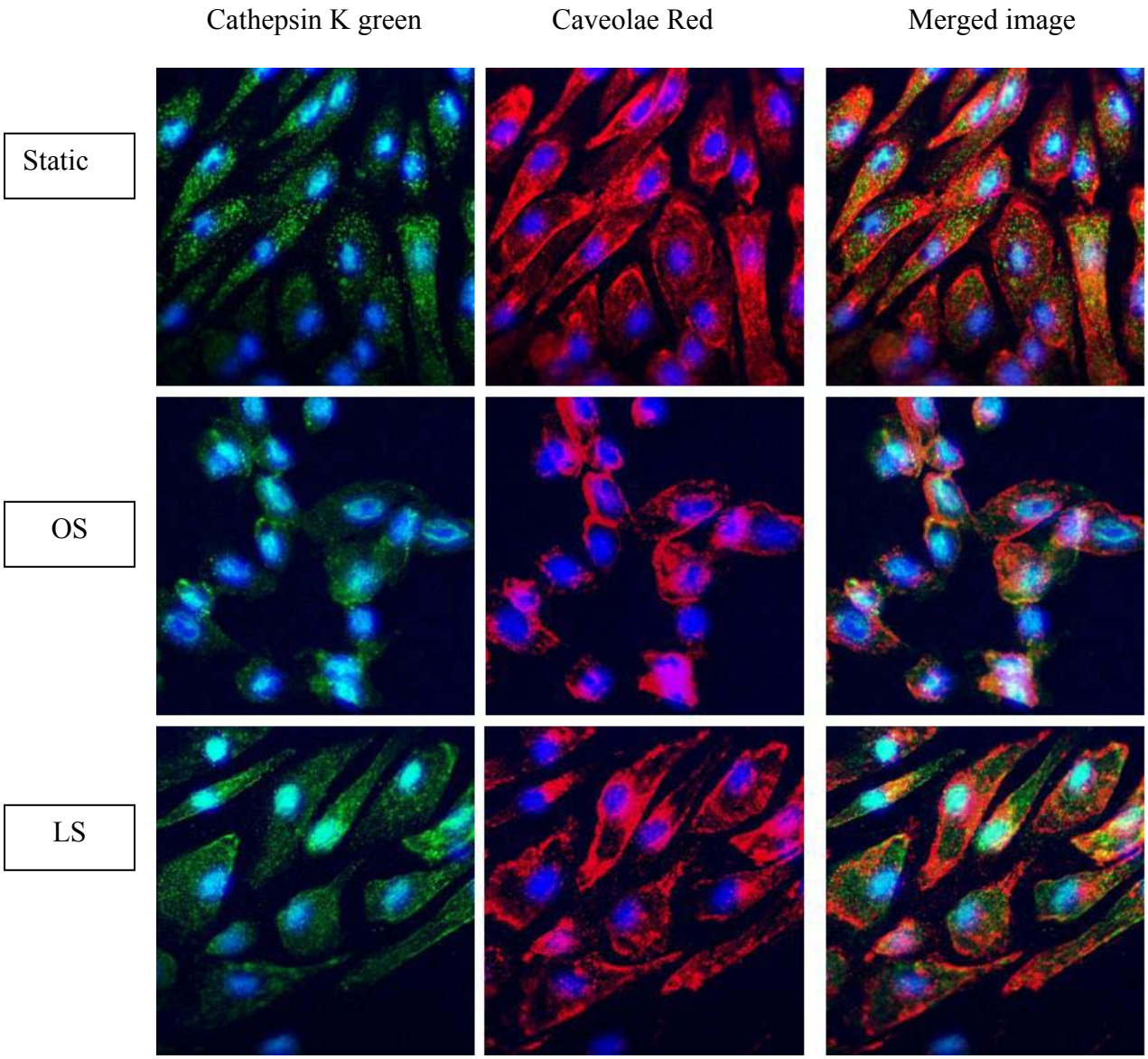

Figure 6.8. Cathepsin K shows some co-localization with caveolin-1. Subcellular proteome extraction and immunolabeling for cathepsin K and caveolin-1 after extraction of the cytosol (Static) and the membrane and organelle fractions (OS and LS). Cathepsin K-green, caveolin-1-red, yellow-merged.

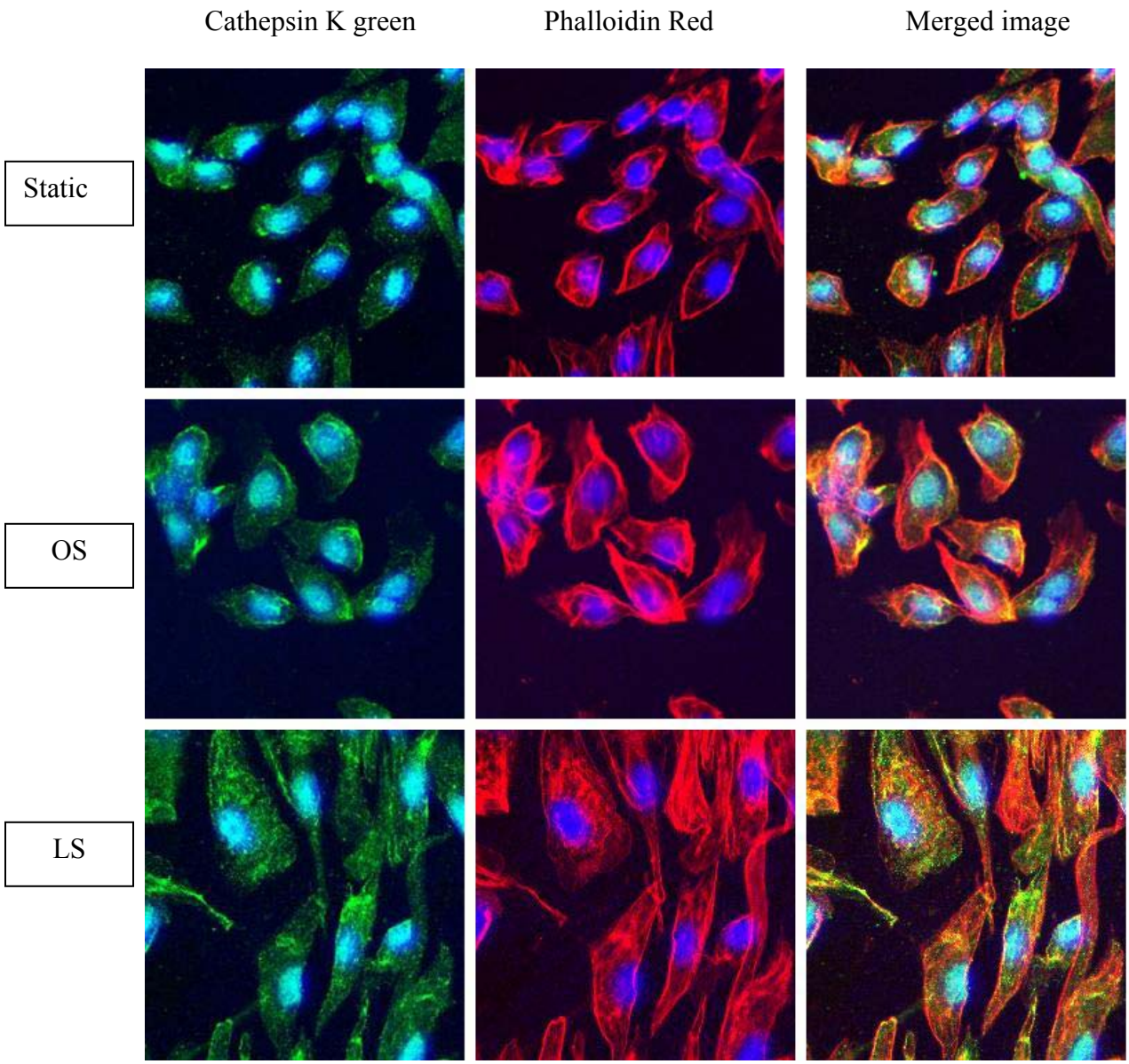

Figure 6.9. Cathepsin K shows co-localization with filamentous actin. Subcellular proteome extraction and immunolabeling for cathepsin K and rhodamine phalloidin labeling after extraction of the cytosol and membrane and organelle fractions. Cathepsin K-green, filamentous actin-red, yellow-merged.

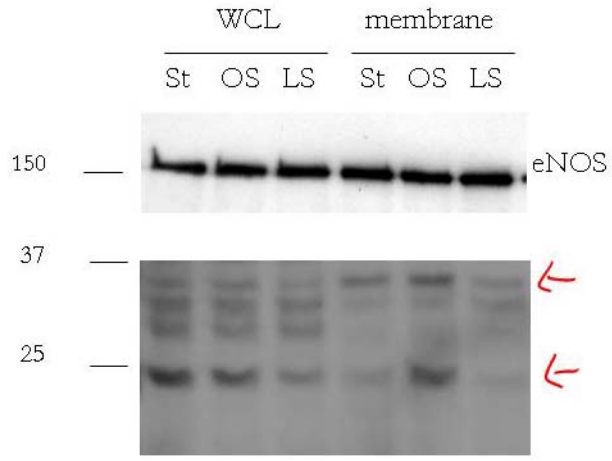

Figure 6.10. Cathepsin K is found in the cell membrane of MAEC. After shear exposure, plasma membrane fraction of MAEC were isolated and run for Western blot. Representative blot of three independent experiments is shown. (WCL – whole cell lysate)

Using more specific techniques to detect cathepsin K in different areas of the cell, we performed cell fractionation to isolate the plasma membrane. MAEC exposed to 24 hours OS, LS, or no flow were lysed and fractioned on an Optiprep gradient. Equal aliquots of protein from plasma membrane fractions were prepared for Western blotting and probed for cathepsin K. In the whole cell lysate, we detected cathepsin K with the same regulation by shear as seen earlier; highest in static, then OS, and the least intracellular cathepsin K in those exposed to LS. Interestingly, the plasma membrane fractions showed a change in relative amounts of cathepsin K with OS having the most amount of the enzyme in its plasma membrane fraction (Figure 6.10). We also used a Versene wash on cells exposed to OS, LS, or no flow to determine if cathepsin K was bound to the surface by a calcium-dependent mechanism as annexin II is known to be (20). There was no detectable cathepsin K in the Versene wash (Figure 6.11A) although

we did detect annexin II (Figure 6.11B) leading us to conclude that cathepsin K is not bound in a calcium dependent manner.

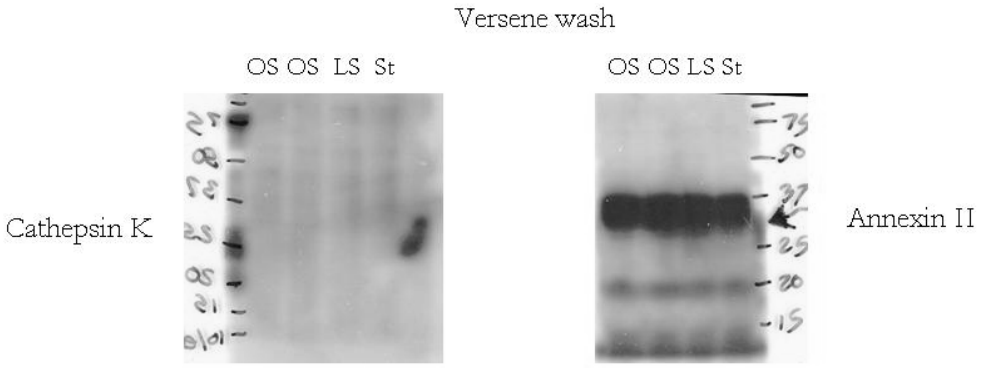

Figure 6.11. Cathepsin K is membrane bound, but not by a calcium dependent mechanism. MAECs exposed to shear were lysed in buffer A and fractionated on an Optiprep gradient to collect plasma membrane, and equal amounts of protein were probed for cathepsin K. MAECs exposed to shear were rinsed with a Versene wash (PBS + 0.53 mM EDTA) and collected protein was probed for A) cathepsin K and B) annexin II.

Discussion

In combination, these studies show that cathepsins L and S have a predominantly lysosomal location within MAEC, but cathepsin K differs. We have seen cathepsin K in the cytoplasm and associated with membrane and cytoskeletal structures both by immunolabeling and molecular biological techniques, and are still detecting its activity in acidic compartments inside of the cell. We further show that cathepsin K shows some co-localization with caveolin-1, a marker of caveolae and also co-localizes with actin on the cell periphery by immunolabeling after subcellular proteomic extraction. The first step of the extraction removes the loosely bound vesicular structures found in the control labeled cells.

Cathepsin K is secreted by osteoclasts at the ruffled border where it serves to resorb bone in the acidic lacunae there (5, 6, 21, 22). ECs secrete cathepsin K also, but

may also bind it to membrane for local proteolysis of basement membrane or other structures as needed in response to disturbed flow as found at sites of vascular remodeling leading to plaque formation and aneurysmal development. In *in vivo* studies, cathepsin K has been co-localized with ECs, SMCs, and macrophages in diseased vessels (23). It was also found at sites of plaque rupture; ECs with cathepsin K bound at their cell surface at regions of disturbed flow could have activated cathepsin K, a powerful collagenase (24), where it is capable of degrading the collagen-filled fibrous cap and cause plaque rupture. More work needs to be done to test this hypothesis.

Cathepsin S has recently been shown to bind to integrin alpha V beta 3 on the smooth muscle cell surface resulting in a staining pattern similar to what we have seen with cathepsin K on the EC surface (12).

It is possible that cathepsin K is associating with an integrin as well which places it at a prime location to participate in cell migration and proteolysis of ECM components. Integrin alpha V beta 3 is implicated by numerous studies in cell migration and angiogenesis and also binds MMP-2 at a non-RGD sequence that is still to be determined (25). Further investigations should also consider cathepsin K in these processes.

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CHAPTER 7

UNIDIRECTIONAL LAMINAR SHEAR STRESS INCREASES CYSTATIN C IN ENDOTHELIAL CELLS

Introduction

Cathepsins B, K, L, and S are highly elastinolytic and collagenolytic cysteine proteases, capable of degrading the main structural components of the blood vessel wall (1-12). Our work has shown that oscillatory shear stress (OS), such as that found in regions of disturbed flow where atherosclerotic plaques form, increases their activity and secretion from endothelial cells. Conversely, unidirectional laminar shear stress (LS) inhibits their synthesis and activity in endothelial cells. The acidic environment created by macrophage secretions in the plaque-forming, intimal region would provide an adequate environment for cathepsin activity although cathepsin S is still active at a neutral pH (1). Release of these proteases into the intimal region of the vessel wall demands that an inhibitor be present to keep their activity at a minimum and to avoid a pathological condition; cystatin C is such an inhibitor.

Cystatins are the group of inhibitors of lysosomal cysteine proteases, of which, cystatin C is the most powerful (1). This 13 kDa protein is translated with a 26 amino acid signal peptide that targets it for secretion approximately an hour after post-translational processing (2). At this point its three-dimensional conformation forms a wedge shape that will block the substrate binding cleft of the cathepsin in a tight, reversible fashion (3) with an inhibition constant on the subnanomolar range (4). In the context of atherosclerosis, its production and secretion has been studied in smooth muscle cells of blood vessel walls. TGF-β, a cytokine present in blood vessel walls, induces an

increased secretion of cystatin C from smooth muscle cells although there is no detectable change in its mRNA levels (5). Cystatin C has also been found in normal blood vessel tissue sections in the medial layer, but was absent in atherosclerotic sections (5, 6). Mice null for cystatin C and Apolipoprotein E fed a high fat diet had increased elastic lamina degradation, increased smooth muscle cell and collagen content in the intimal region, and had expanded abdominal aortas providing evidence of the importance of this protease inhibitor *in vivo* in preservation of elastic structures in the arterial wall (7). Its production by endothelial cells remains elusive. We show here that cystatin C is upregulated by endothelial cells in response to unidirectional laminar shear stress and downregulated under an oscillatory shear stress yet need to further investigate the molecular roles of cystatin C in atherosclerosis.

From this data, we hypothesize that laminar shear stress induces the production and secretion of cystatin C by endothelial cells, and that these levels regulate and inhibit the vessel wall remodeling activities of cysteine proteases. Alternatively, oscillatory shear stress signals endothelial cells to reduce either their production and/or secretion of cystatin C, while increasing cathepsin expression. This results in a shift in the balance between the proteases and their inhibitor. Eventually, this results in vessel wall remodeling and increased atherosclerotic plaque formation and growth.

Methods

Mouse Aortic Endothelial cells (MAEC) culture and shear stress studies. MAEC obtained from the thoracic aortas of C57/BL6 mice were isolated, cultured in growth medium [Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine

serum (FBS), 100 μg/ml endothelial cell growth supplement (ECGS, Sigma) and 2.5 U/ml heparin] as described (8), and used between passages 7-10. Confluent endothelial monolayers grown in 100-mm tissue culture dishes were exposed to an arterial level of uni-directional LS (15 dyn/cm^2) or OS with directional changes of flow at 1 Hz cycle (± 5 dyn/cm^2) for 1 day by rotating a Teflon cone (0.5° cone angle) as described previously (8). One hour prior to shear, the monolayers were washed and changed to 10 ml of fresh shear medium (the growth medium without serum).

Immunocytochemistr*y.* Cells were rinsed with PBS, and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS, and the fixative was quenched with 50 mM ammonium chloride. Blocking buffer of 3% BSA in PBS was preincubated with the cells for an hour, followed by overnight incubation with the primary antibody: anti-cystatin C (1:100, Upstate), anti-GM 130 (1:100), anti-vinculin (1:100, Calbiochem). Alexa 488 (green) or Alexa 568 (red) conjugated secondary antibodies were used to fluorescently label the primary antibody. Cells were rinsed with PBS then covered with Prolong anti-Fade Kit (Molecular Probes) and visualized on an epifluorescent microscope.

Immunohistochemistry*:* Frozen sections of human coronary arteries from transplant patients were acquired and fixed in acetone. After air drying and blocking for 30 minutes with 1% gelatin/PBS, the cathepsin K primary antibody (Calbiochem) was incubated in a 1:20 dilution of 1% BSA/PBS for one hour followed by a PBS rinse then secondary antibody incubation, conjugated to alkaline phosphatase, diluted in 1% BSA/PBS + 2%

normal serum for 30 minutes. Vector ABC kit (Vector) was used for color reaction according to manufacturer's instructions. Hematoxylin was used to counterstain. Negative control was incubated with 1% BSA/PBS in the absence of primary antibody. Slides were viewed with a microscope to visualize color reaction, and then viewed under an inverted fluorescent microscope with FITC filter and laser excitation to view elastin auto-fluorescence.

Microarray. Total RNA was collected from MAEC after 24 hours of OS, LS, or Static culture and transcribed into cDNA, linearly amplified to cRNA, and hybridized to a Codelink DNA microarray. Fluorescent labeling of bound cRNA was scanned for intensity.

Enzyme Linked Immunosorbent Assay (ELISA). Assay was performed according to manufacturer's instructions (Abcam). Briefly, serum free conditioned media was collected after 24 hours OS, LS, and St and normalized to 10 ml, the pre-shear volume. Equal aliquots of 100 µl of conditioned media or cystatin C standards were dispensed into wells pre-coated with anti-cystatin C antibody. Wells were washed thoroughly then incubated with horseradish peroxidase (HRP) labeled anti-cystatin C antibody. After washing again, tetramethylbenzidine substrate was added to react with bound HRP and acidic stop solution (0.2 M H_2SO_4) was added to stop the yellow product forming. This color change was measured with a spectrophotometer at 450 nm, and media samples were fit to the standard curve.

Plasmid transfection. Full length cystatin C on a CMV Sport6 plasmid (ATCC) was transfected into subconfluent MAEC with Lipofectamine (InVitrogen) in serum free medium. After 6 hours, the medium was supplemented with serum (final 10% concentration) and cultured an additional 42 hours prior to exposing the cells to OS, LS, or no flow conditions.

Cathepsin activity assay. Conditioned media were collected and concentrated. Aliquots were added to a reaction mixture containing 100 mM acetate, pH 5.5, 2.5 mM EDTA, 2 mM dithiothreitol, and 0.1% Brij 35. Benzyloxycarbonyl-Gly-Pro-Arg-7-amino-4-methylcoumarin (Z-GPR-AMC) (Biomol) was used as the substrate and added to attain a final concentration of 20 μM after the cathepsins were activated for two minutes at 37°C (9). The reaction mixture was incubated at 37°C for 60 minutes, and AMC fluorescence intensity was determined with a fluorescence plate reader (excitation at 360 nm and emission at 460 nm).

Western blots. Following shear exposure, conditioned media were collected and normalized to 10 ml total with fresh serum free shear media if necessary, and concentrated 20 to 30 fold with a spin concentrator (5 kDa molecular weight cutoff, Vivascience) or precipitated in five times the volume of ice cold acetone then centrifuged and resuspended at a 20 fold concentration of lysis buffer. Cells were rinsed twice with phosphate buffered saline and then lysed with RIPA buffer. Following modified Lowry protein assay, equal amounts of total protein were resolved by SDS-PAGE. Protein was transferred to a polyvinyldifluoride membrane (Millipore), and probed with a rabbit

polyclonal anti-cystatin C antibody (1:1000, Upstate), anti-cathepsin K (1:250, Calbiochem) and a secondary antibody conjugated to alkaline phosphatase (Bio-Rad), which were detected by a chemiluminescence method (10).

Results

Laminar shear stress upregulates cystatin C mRNA and protein in mouse aortic endothelial cells.

MAECs were grown to confluence and exposed to 24 hours of OS, LS, or St culture as described above. Total RNA was collected and used for DNA microarray analysis on the Codelink array. LS significantly increased cystatin C mRNA level by 2.5 fold over OS and St as determined by the normalized fluorescent intensity on the array (Figure 7.1A). Western blots were used to illustrate that the full protein is being expressed and secreted by the endothelial cells, in a manner that increases and decreases with mRNA levels. Media was collected following a 24 hour shear period, and the proteins were precipitated with acetone. After resuspension in sample buffer containing SDS and beta-mercaptoethanol, equal volumes were loaded into a 12.5% SDS-PAGE gel and blotted for cystatin C. Cystatin C is largely secreted into the media during laminar shear stress, but at low levels under oscillatory shear (Figure 7.1B); it was undetectable in the cell lysate in any of the shear conditions. ELISA performed on the conditioned media confirmed 3 fold higher cystatin C produced by the cells exposed to LS over OS (Figure 7.1C).

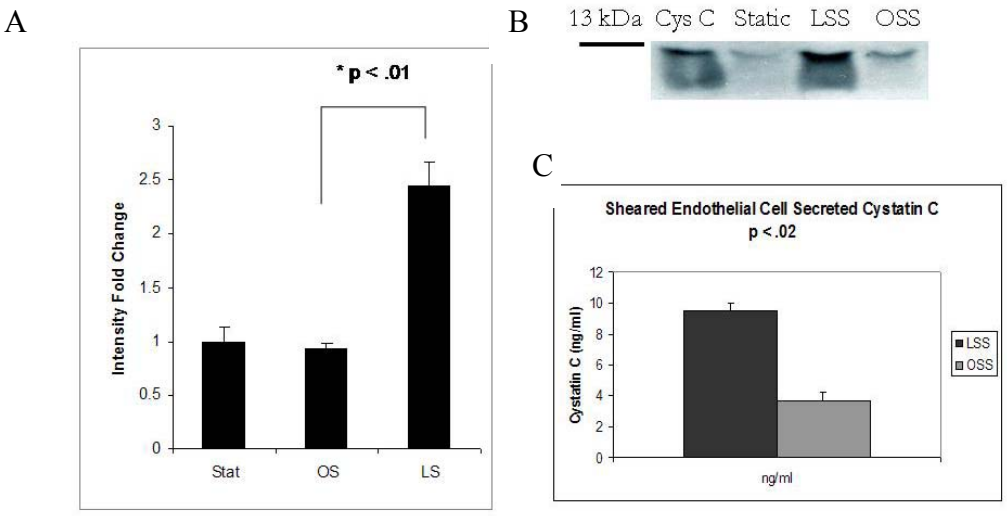

Figure 7.1. **LS upregulates cystatin C mRNA and secreted protein in MAEC.** Confluent MAEC were exposed to 24 hours OS, LS, or St culture, and total RNA and conditioned media were collected. A) Total RNA was transcribed into cDNA, linearly amplified to cRNA, and hybridized to a Codelink DNA microarray. Fluorescent labeling of bound cRNA was scanned for intensity. Bar graph shows median normalized values as fold change of Static control (n=3). B) Conditioned media was precipitated with acetone and equal aliquots of protein or cystatin C positive control were prepared for Western blot with anti-cystatin C antibody. C) Dilutions of conditioned media were used for ELISA assay according to manufacturer's instructions and compared to a standard curve.

LS correctly processes cystatin C through the secretory pathway but OS does not.

To visualize cystatin C intracellularly, since it was not detected in the Western blot, we performed immunocytochemistry on the MAEC after exposure to shear and found that the intracellular location of cystatin C changed according to the type of shear stress. Under OS and St culture, the cystatin C was diffuse in the cell and does not co-localize with GM-130, the cis-Golgi marker (Figure 7.2). However, there is a marked difference when the MAEC were exposed to LS; cystatin C is highly colocalizing with the Golgi apparatus, an organelle responsible for packaging secreted proteins. This suggests that some mechanism induced by LS leads to the proper packaging of cystatin C

for secretion into the extracellular environment, but this mechanism is not turned on in response to OS or static culture where you see higher production and secretion of cathepsins.

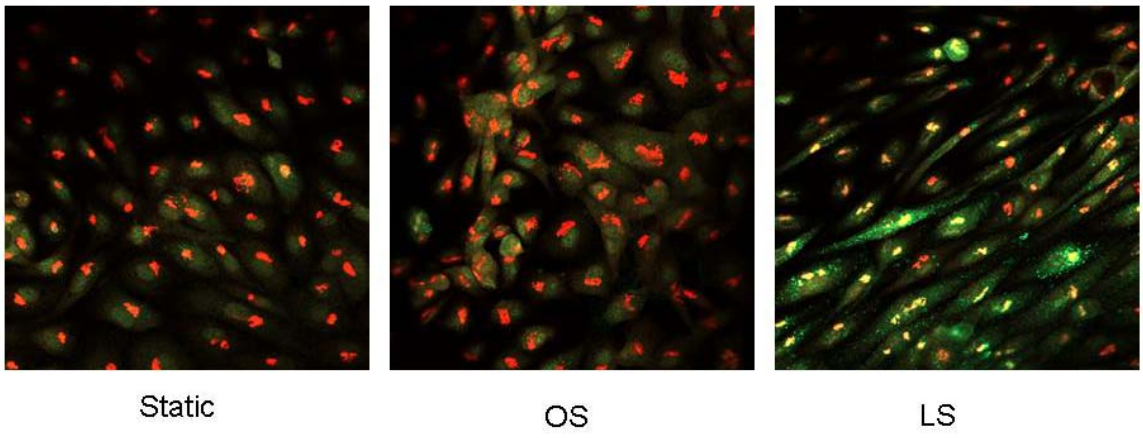

Figure 7.2. LS correctly processes Cystatin C through secretory pathway but OS does not. Confluent MAECs exposed to 24 hours OS, LS, or St were fixed with 4% paraformaldehyde and permeabilized before labeling with anti-cystatin C antibody (green) and anti-GM130 for cis-Golgi (red). Co-localization is seen with the yellow.

Since our hypothesis requires that cystatin C be secreted into the vessel wall to inhibit any cathepsins present there, it became important to identify the polarity of the secretion of this inhibitor. Again, we used confocal microscopy to reconstruct a three-dimensional picture of the endothelial cells and cystatin C. Using vinculin, a focal adhesion protein, as a marker for the basal cell surface, MAEC were immunolabeled with anti-cystatin C (Figure 7.3A) and anti-vinculin (Figure 7.3B) antibody and after exposure to 24 hours of OS, LS, or St culture. Hoechst was used to label cell nuclei. Then, a three-dimensional projection was constructed from the Z-series of slices above and below the endothelial cell height. From these images, it can be seen cystatin C (red) is co-localizing in that basal region of the cell with the vinculin (green) suggesting its targeting out of the basal side into the basement membrane and that the nuclei sit above the

cystatin C (Figure 7.3D). One can conjecture that if these cells were lining the arterial wall *in vivo*, then the protein would be secreted into the artery and not completely out of the luminal side.

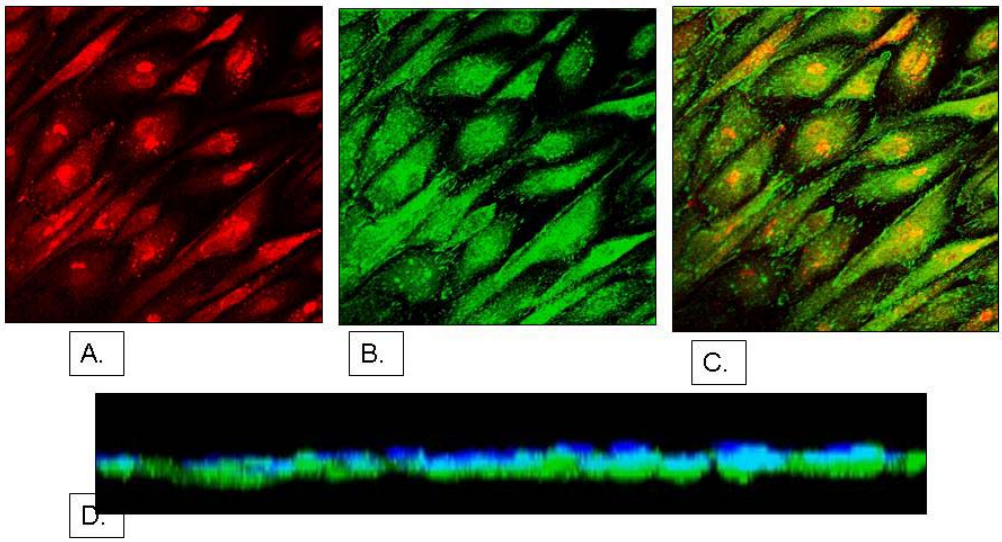

Figure 7.3. Endothelial cells show packaging and basal secretion of cystatin C due to LS. Immunostaining for cystatin C (A, red) and vinculin (B, green), a marker for focal adhesions. Here we see that cystatin C is colocalizing with the basal cell marker (C). Projection (D) of sheared endothelial cells shows blue Hoechst stained nucleus above the cystatin C (Green here) additionally supporting its basal location.

LS conditioned MAEC uptake more cystatin C from the extracellular environment.

To block shear stress mediated, cathepsin dependent elastase activity, 0.5 μg/ml cystatin C was added to MAEC after exposure to shear with BODIPY-elastin. Native cystatin C did not decrease elastase activity but increased it by 40%, 25%, and 28% in the LS, OS, and St cases, respectively over the boiled cystatin C samples (Figure 7.4A).

It was also interesting to note that when MAECs were incubated with excess cystatin C in the presence of soluble elastin, it was detectable in the cell lysate by Western blots suggesting a mechanism by which cystatin C is endocytosed from the

media, or the blood. There was a preferential uptake of cystatin C by those cells exposed to LS, and not because there was more cystatin C present since fresh media containing equal amounts of the protein was added to each (Figure 7.4A). There was no preferential uptake of cystatin C when it was denatured by boiling, but an equal, general uptake under OS, St, and LS exposed MAEC (Figure 7.4B). Those not incubated with the boiled cystatin C still did not have detectable levels in their cell lysates (Figure 7.4C). Taken together, these results suggest that there is a mechanism by which cystatin C is selectively endocytosed by endothelial cells under high laminar shear stress conditions and may be able to transcytose it into the vessel wall whether they synthesized it themselves or not.

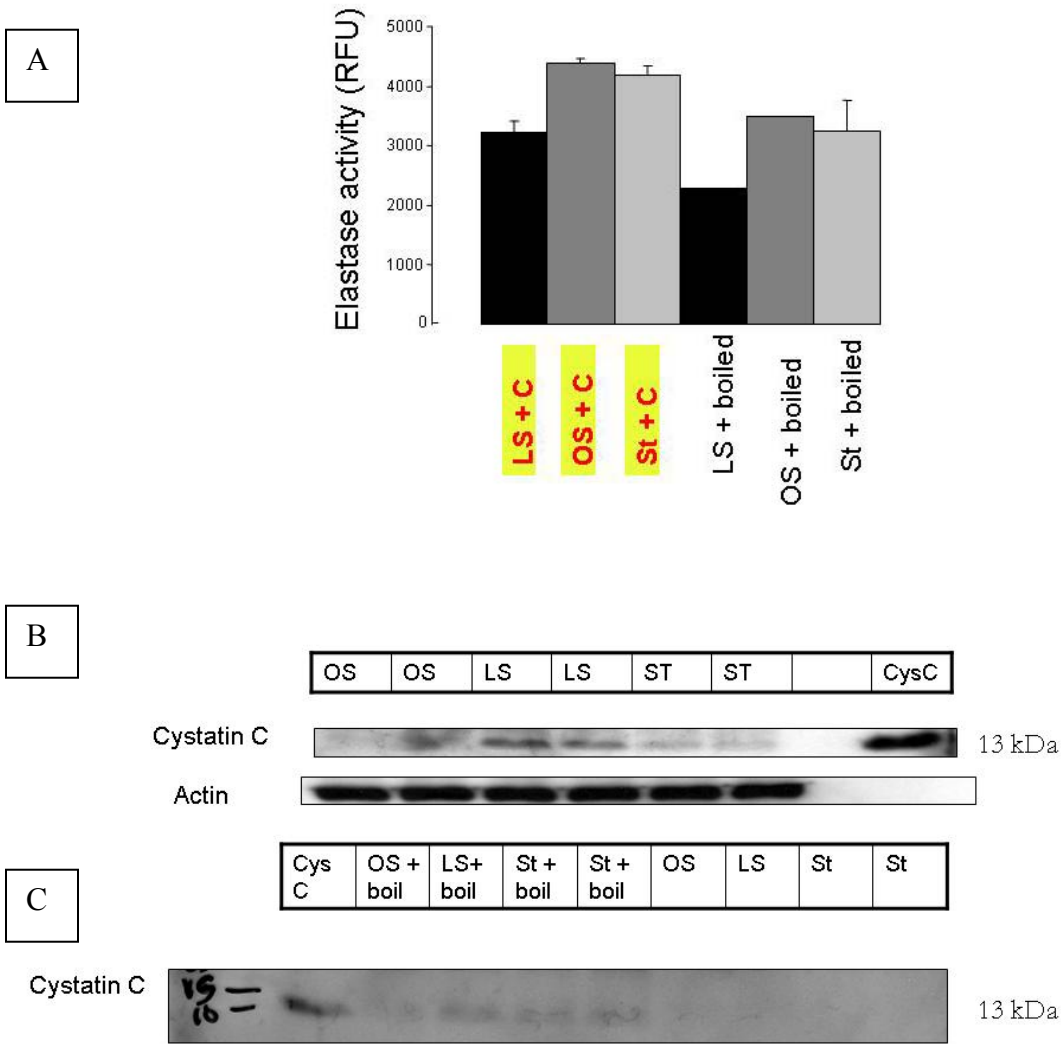

Figure 7.4. LS conditioned MAECs uptake more cystatin C than St or OS conditioned MAECs. Confluent MAECs, after exposure to 24 hours of OS, LS, or St were incubated with either native or boiled 0.5 µg/ml cystatin C in the presence of BODIPY labeled soluble elastin. Elastase activity is shown in (A) and W. blots of cell lysates are shown after incubation with native cystatin C (B) or denatured cystatin C (C).

Endothelial cells overlying atherosclerotic regions reduce cystatin C production.

To determine if cystatin C was important *in vivo* in the development of atherosclerosis, we performed immunohistochemical staining of human coronary arteries at different stages of atherosclerotic development and paid particular attention to the

endothelial cell staining of cystatin C. It was previously reported by Shi et al that cystatin C levels are decreased in atherosclerotic vessels and our findings corroborate that. Additionally, it can be seen that the endothelium of the diseased vessel is not expressing cystatin C protein at all while the minimally diseased vessel stains strongly in the endothelial cell layer (Figure 7.5A, B). Staining with von Willebrand factor shows the presence of endothelium in both vessels. (Figure 7.5C, D)

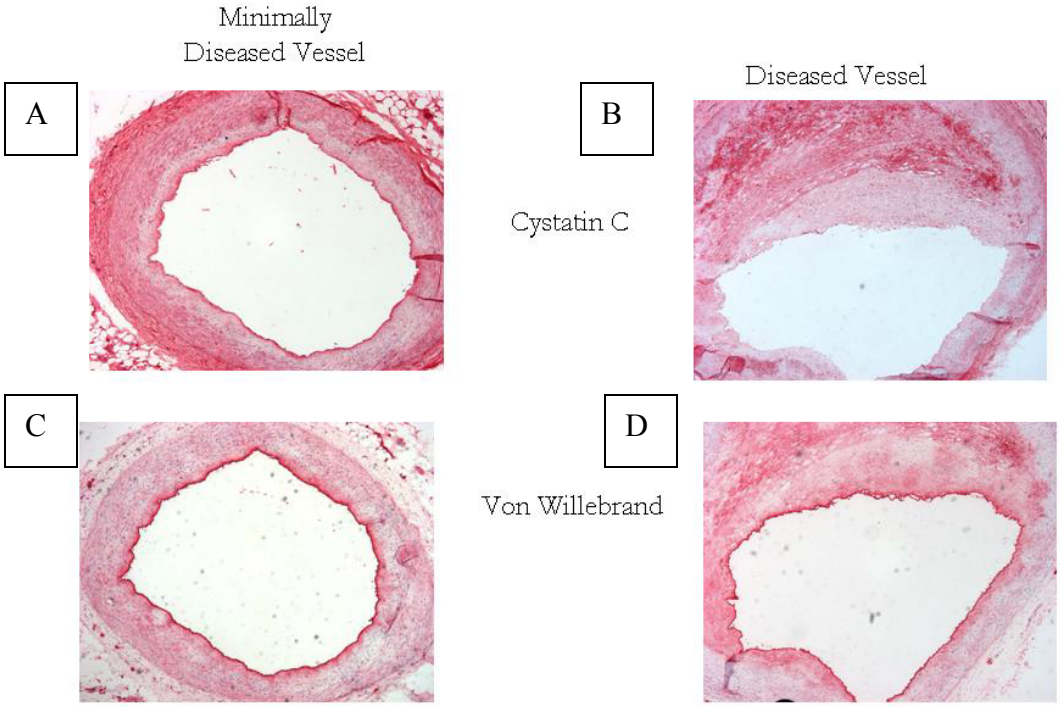

Figure 7.5. Cystatin C immunohistochemistry. Frozen sections of minimally diseased or atheromatous human coronary artery samples from patients receiving heart transplants were stained with anti-cystatin C antibody (A, B) or anti-von Willebrand factor (C, D).

Binding cystatin C increases cathepsin activity.

It still remained to be shown that cystatin C was inhibiting cathepsin activity extracellularly in response to LS. To do this, we used carboxymethylated papain as a sort of "inhibitor" for cystatin C. Cystatin C has a tight binding affinity for papain (from

which the name papain family of cysteine proteases was given to the cathepsins) but when papain is carboxymethylated, it loses its proteolytic activity but not its affinity for cystatin C. Agarose beads coated with carboxymethylated papain (CM-papain) were sheared with MAEC under OS, LS, and St conditions and the conditioned media was collected to be used for Western blot and cathepsin activity assays. As seen in figure 7.6A, MAEC still elongate and align in the direction of flow with the beads present and are not scraped off of the plate. Western blotting of the conditioned media confirms that the CM-papain does indeed bind the free cystatin C secreted by the endothelial cells (Figure 7.6B) during the shear stress exposure but does not bind cathepsin K, a prime target for cystatin C (Figure 7.6C).

The conditioned media was then centrifugally concentrated and equal aliquots of protein were used for cathepsin activity assay with the fluorogenic, synthetic substrate Z-GPR-AMC. Figure 7.6D shows that without the CM papain beads, OS increases cathepsin activity by almost two-fold of the static and LS groups. When the free cystatin C is bound to the CM papain beads the cathepsin activity under LS increases significantly and suggests that the reduced level of cathepsins that are present in the conditioned media after culture under LS are usually inhibited by cystatin C; without its binding, they are capable of hydrolytic activity.

Additionally, we used a cystatin C plasmid to overexpress this protein in MAEC as seen in the conditioned media Western blots of figure 7.7. Under OS, overexpressing cystatin C reduced Z-GPR-AMC hydrolysis to equal levels with the St and LS groups, again illustrating that cystatin C is capable of inhibiting extracellular cathepsins.

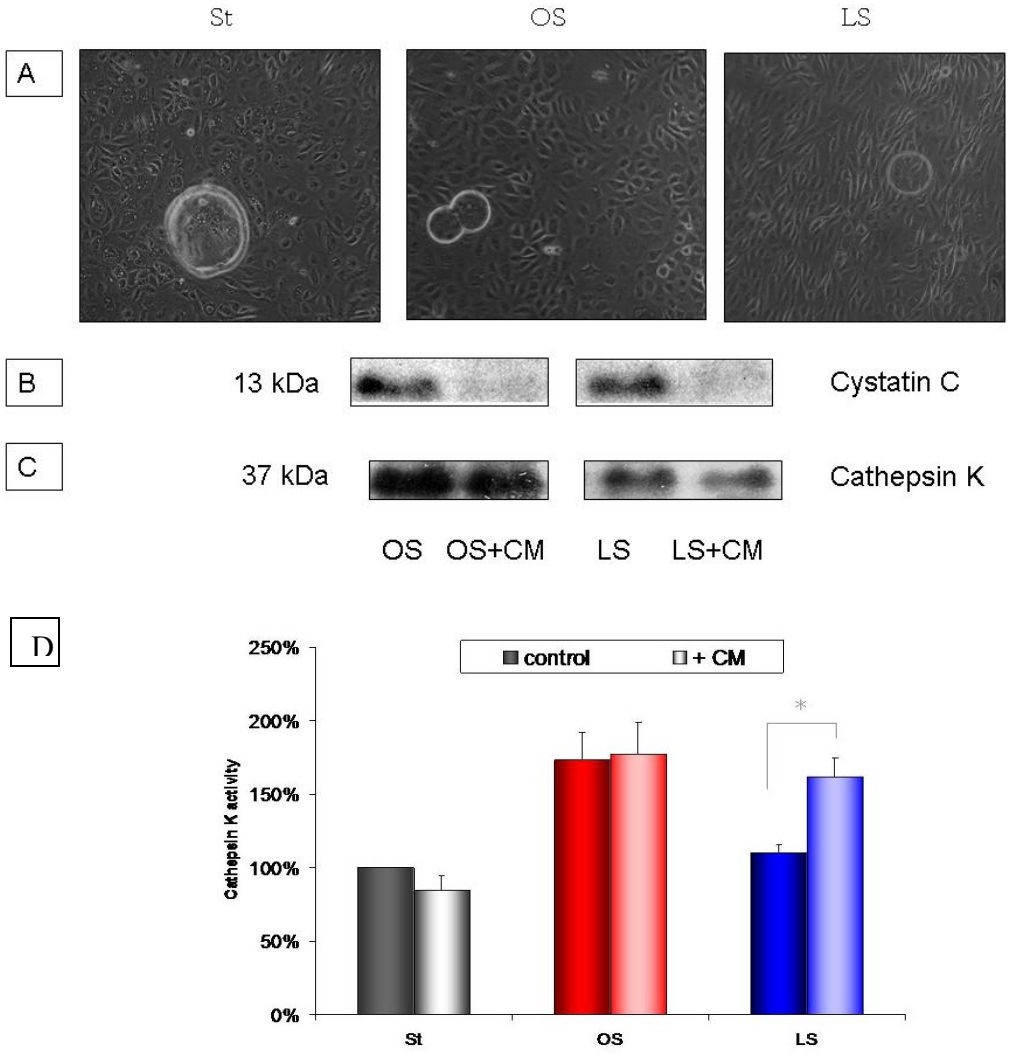

Figure 7.6. CM-papain beads bind cystatin C and increase cathepsin activity. Confluent MAECs were exposed to 24 hours of OS, LS, or St either with or without (9 ng/ml) of carboxymethylated (CM) papain bound to agarose beads in serum free media and the conditioned media was collected and X protein was precipitated for W. blot or centrifugally concentrated for cathepsin activity assays (D). Bar graph shows Z-GPR-AMC hydrolysis as % of static control (n=3, * p<.05).

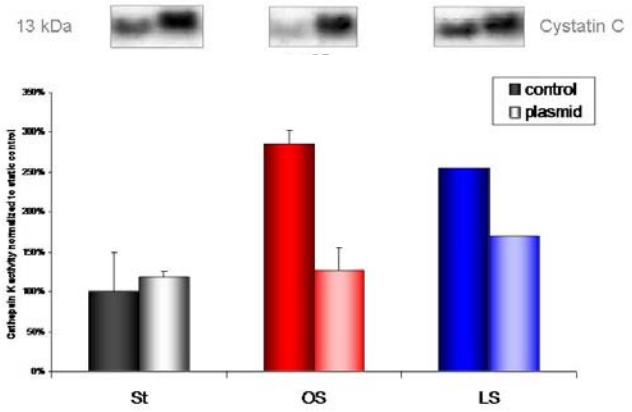

Figure 7.7. Overexpression of cystatin C reduces OS mediated cathepsin activity. MAEC transfected with cystatin C plasmid under constitutive CMV promoter were exposed to 24 hours OS, LS, and St conditions. Conditioned media was collected for W. blot and cathepsin activity assay. Bar graphs show Z-GPR-AMC hydrolysis as % of static control (n= 2 to 3).

Discussion

Remodeling of the blood vessel wall is regulated by proteinases and their endogenous inhibitors. Cystatin C, thhe most powerful endogenous inhibitor of the papain family of cysteine proteases, has been shown here to be regulated by shear stress in that 1) LS increases cystatin C mRNA and protein levels 2) LS causes cystatin C to be processed intracellularly through the Golgi apparatus for secretion while OS and St do not 3) Cystatin C seems to accumulate in the basal portion of the endothelial cell 4) Endothelial cells are capable of taking up cystatin C from the extracellular environment 5) Endothelial cells overlying atherosclerosis do not express cystatin C, but those in the normal vessel do 5) binding free cystatin C increases cathepsin activity and 6) overexpressing cystatin C decreases cathepsin activity. Together, these results indicate that cystatin C is atheroprotective in the vessel wall where it serves to inhibit cathepsin proteolytic activity.

Cystatin C is a constitutively secreted protein by many different tissues with an intracellular half life of about 72 minutes (2), but here we are showing regulation of cystatin C protein levels by shear stress. Promoter studies of cystatin C show that it has constitutive elements AND regulatory elements (11). There are 11 Sp1 binding sites that may serve regulatory function (11) and Sp1 is known to be a shear responsive element that is phosphorylated due to LS (12-14). Additionally, there is a GATA box in the promoter, and these are known to regulate endothelial cell expressed genes (11). Promoter analysis therefore supports the results that shear stress on endothelial cells regulates cystatin C transcriptional activity and is verified with our DNA microarray (Figure 7.1A). The protein studies corroborate the microarray with increased cystatin C protein after LS exposure on endothelial cells (Figure 7.1B).

Cystatin C is elusive to detection intracellularly without cellular manipulation but we could label it fluorescently with antibodies *in situ*. The finding that LS promotes proper packaging of cystatin C through the Golgi apparatus for secretion into the extracellular space (Figure 7.2) supports the increased protein level found in the conditioned media after LS exposure. Normally, cystatin C forms an inactive dimer in the endoplasmic reticulum, is packaged for secretion in the Golgi, and is then secreted as a monomer (2). Alterations to the cystatin C sequence can change this sequence of events. The cystatin C gene without the leader sequence that targets it for secretion was transcribed and translated in retinal epithelial cells, was not secreted, and did not go through the Golgi apparatus (15). In fact, immunostaining of the cells closely resemble the pictures presented here under OS and St conditions with diffuse cystatin C staining throughout the cell in comparison to sharp localization in the Golgi apparatus of the cells

exposed to LS or transfected with the cystatin C DNA containing the leader sequence. Since the cystatin C promoter has heterogeneous start sites and minor start sites (11), there may be alternative transcription under OS or St conditions that do not lead to the appropriate mRNA transcript with the leader sequence.

Cystatin C localizing to the basal portion of the endothelial cell supports the findings of other groups that normal cystatin C is secreted basally (16). Immunohistochemistry of human coronary arteries for cystatin C showed a strong staining in the endothelium of the minimally diseased vessel, but a lack of endothelial cell staining in the vessel with the atherosclerotic plaque (Figure 7.5A, B). It has been shown previously that atherosclerotic vessels do not contain cystatin C but normal, healthy vessels do (17). Our staining serves to corroborate this evidence and to add the loss of cystatin C staining of the endothelium. We are not sure whether these sections were at sites of disturbed flow *in vivo* before being removed for sectioning, but extrapolating from the accepted notion that atherosclerosis occurs at sites of disturbed flow, we can assume that the more diseased vessel was at a region of disturbed flow. This disturbed flow, or oscillatory shear stress, would have decreased endothelial cell production of cystatin C according to our *in vitro* results presented here.

Binding of free cystatin C serves to demonstrate its role in inhibiting cathepsin activity (Figure 7.6). Immunoprecipitation would directly show this binding, but since our antibodies detect both the pro- and mature forms of the cathepsins, of which the proform was more abundant in the conditioned media, this assay could not be used. The carboxymethylated papain beads serve to remove cystatin C from the system allowing the cathepsins to remain unbound and active leading to their increased activity extracellularly

under LS; the low levels of cathepsin present under LS still have activity when not inhibited by cystatin C (Figure 7.6D). There was no increase by OS presumably because there was already a lowered level of cystatin C that was outnumbered by the increased cathepsin production stimulated by OS. Overexpression of cystatin C in the endothelial cells reduced the OS mediated activity of the cathepsins, substantiating the idea that addition of cystatin C after OS, when levels are low, is sufficient to inhibit cathepsin activity (Figure 7.7).

In an attempt to use this concept to determine if extra cystatin C could inhibit the shear mediated and elastase activity that we have shown is cathepsin dependent, the results were confounding. Addition of cystatin C did not reduce the elastase activity (Figure 7.4A). Western blots on those cell lysates, for the first time, detected cystatin C intracellularly (Figure 7.4A), and preferentially for native, nondenatured cystatin C under LS (Figure 7.4B); boiled cystatin C was nonspecifically taken up under all shear conditions (Figure 7.4C). This requirement for cystatin C to be in its native conformation suggests that there is some sort of receptor mediated uptake of cystatin C. *In vivo* studies of cystatin C uptake by endothelial cells after intraretinal injection in mice has been previously studied advocating the idea that endothelial cells are capable of taking up this inhibitor (18). It was further shown that this uptake in the retina is an active process that requires energy since lowering the temperature or depolymerizing actin with cytochalasin D reduced the uptake (19). Again, this suggests a receptor mediated process. Cystatin C receptors have not been identified, but there is evidence that it binds to the TGF-β R-II to antagonize its TGF-β signaling (20, 21); there are no reports yet of it being endocytosed by this receptor. Cystatin C circulates in the plasma and it may be that endothelial cells,

aside from producing cystatin C under LS conditions, may also transcytose cystatin C from the plasma and pass it into the vascular wall to increase the concentration there for prevention of vascular remodeling due to cathepsins.

Understanding the atheroprotective mechanisms by which laminar shear stress maintains the integrity of the arterial wall and the elastic structures contained therein can prevent plaque formation. These results show that cystatin C, the powerful inhibitor of the papain family of cysteine proteases that are being implicated in atherosclerosis, is important and that its inverse regulation with cathepsins maintains balance in this system; shear stress and endothelial cells seem to be the mediators of this balance.

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CHAPTER 8

CATHEPSINS, SHEAR STRESS, AND PROGRAMMED CELL DEATH IN ENDOTHELIAL CELLS

Introduction

During the course of studying the role of cathepsin K in shear stress induced elastase and gelatinase activity using siRNA, we noticed a side effect of vacuole formation and unhealthy endothelial cells even under static conditions. It is reported that low or oscillatory shear stress induces apoptosis in endothelial cells (1) and that oxidized low density lipoproteins, which are increased at sites of disturbed flow and in atherosclerotic lesions, also lead to apoptosis in endothelial cells (2). Endothelial cell apoptosis involves the same changes as other apoptotic cells including loss of cell to cell contact, loss of contact with the matrix, movement of cytochrome c to the cytoplasm from the mitochondria, activation of caspases, subsequent Bid cleavage, phosphatidylserine exposure on the external surface of the plasma membrane, a loss of the anti-coagulant properties that make endothelial cell lining of the blood vessel wall necessary, membrane blebbing, and condensation of chromatin (3).

Caspases are the cysteine proteases that trigger and effect the apoptotic pathway in cells. The initiator caspases -2, -8, -9, and -10 activate the effector caspases -3, -6, and -7 that have cleavage targets throughout the cell and in the nucleus to cause the chromatin condensation and membrane blebbing that signal macrophage ingestion of the apoptotic cell *in vivo* and prevent any inflammatory response (4). Cathepsin mediated mechanisms of apoptosis have also been shown to cross paths with the caspases; cathepsin L may also activate caspase-3 (5), and cathepsins B and L were found near TUNEL positive cells in

atherosclerotic lesions and with macrophages that were positive for caspase 3 (6). Additionally, translocation of cathepsins B and D from the lysosome to the cytoplasm is an initiating step in some types of apoptosis and in other forms of programmed cell death (7, 8).

Here we investigated the mechanisms by which transfection of a sequence of siRNA, designed for cathepsin K, but additionally knocks down cathepsins B and L leads to endothelial morphology changes similar to apoptosis or programmed cell death. We also show here that inhibition with a cathepsin K inhibitor induces these similar changes of membrane blebbing and vacuolar formation in mouse aortic endothelial cells and that these changes induce but are not dependent upon caspase activity.

Methods

Mouse Aortic Endothelial cells (MAEC) culture and shear stress studies. MAEC obtained from the thoracic aortas of C57/BL6 mice were isolated, cultured in growth medium [Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 100 μg/ml endothelial cell growth supplement (ECGS, Sigma) and 2.5 U/ml heparin] as described(9), and used between passages 7-10. Confluent endothelial monolayers grown in 100-mm tissue culture dishes were exposed to an arterial level of uni-directional LS (15 dyn/cm^2) or OS with directional changes of flow at 1 Hz cycle (± 5 dyn/cm^2) for 1 day by rotating a Teflon cone (0.5° cone angle) as described previously(9). One hour prior to shear, the monolayers were washed and changed to 10 ml of fresh shear medium (the growth medium without serum).

Transfection of siRNA. To knockdown mouse cathepsin K mRNAs, the annealed siRNA duplex [sense: 5'- AAG GAU ACG UUA CUC CAG UCA tt', antisense: 5'- UGA CUG GAG UAA CGU ACU CC tt] and nonsilencing duplex [sense: 5'- UUC UCC GAA CGU GUC ACG Utt, antisense: 5'- ACG UGA CAC GUU CGG AGA Att] were purchased from Qiagen. Subconfluent (75-80% confluency) MAEC were transfected at a final siRNA duplex concentration of 100 nM using Oligofectamine (Invitrogen) in serum free medium. After 6 hrs, the medium was supplemented with fetal bovine serum (final 10% concentration) and cultured an additional 42 hrs prior to exposing the cells to either OS, LS, or no flow conditions.

Elastase assay. Five μg/ml of BODIPY® fluorescein-conjugated DQ™ elastin (Molecular Probes) in fresh serum-free DMEM was incubated with MAEC following exposure to OS, LS, or St for one day or in six well plates under static conditions in the absence or presence of 50 μM E64 (Sigma), 50 μM GM6001 (Calbiochem), 50 μM of the cathepsin K inhibitor, 1,3-Bis(CBZ-Leu-NH)-2-propanone, (Calbiochem), or the vehicle. After an additional 24 hours, aliquots (200 μl) of conditioned media were assayed with a fluorescence plate reader, in triplicate, with background fluorescence subtracted from the no-cell negative control at 485 nm excitation and 525 nm emission.

Annexin V-FITC Apoptosis detection. Kit was used according to manufacturer instructions. Briefly, 48 hours after transfection with nonsiRNA or cathepsin K370 siRNA, cells were trypsinized and resuspended in media. Media binding reagent was added to 500 μl of cell suspension followed by 1.25 μl Annexin V-FITC ad incubated in

the dark for 15 minutes. Cells were pelleted at 100 x g for 5 minutes ad resuspended in 1X binding buffer. Propidium iodide was then added to the samples. Samples were analyzed by flow cytometry detecting FITC on FL1 detector at 518 nm and detection of propidium iodide on FL2 at 620 nm.

Caspase 3/7 activity assay. Apo3 HTS Apoptosis kit (Cell Technology) was used according to manufacturer instructions. Briefly, after shear or culture in 6 well plates, cells were scraped in PBS and lysed with the proprietary lysis buffer supplemented with 15 mM fresh DTT and the caspase reagent, quenched bisubstituted Z-DEVD R110 Dye reagent. After cleavage by caspases, the R110 Dye is free and fluorescence is read with a spectrofluorometer excitation 488 nm and emission 525 nm in a 96 well plate and normalized to total protein.

Cathepsin activity assay. Cells were lysed in 40 mM sodium acetate buffer, pH 5.5, .1% Triton-X 100, and conditioned media were collected and concentrated. Aliquots were added to a reaction mixture containing 100 mM acetate, pH 5.5, 2.5 mM EDTA, 2 mM dithiothreitol, and 0.1% Brij 35. Benzyloxycarbonyl-Gly-Pro-Arg-7-amino-4-methylcoumarin (Z-GPR-AMC) (Biomol) was used as the substrate and added to attain a final concentration of 25 μM after the cathepsins were activated for two minutes at 37°C (10). The reaction mixture was incubated at 37°C for 60 minutes, and AMC fluorescence intensity was determined with a fluorescence plate reader (excitation at 360 nm and emission at 460 nm).

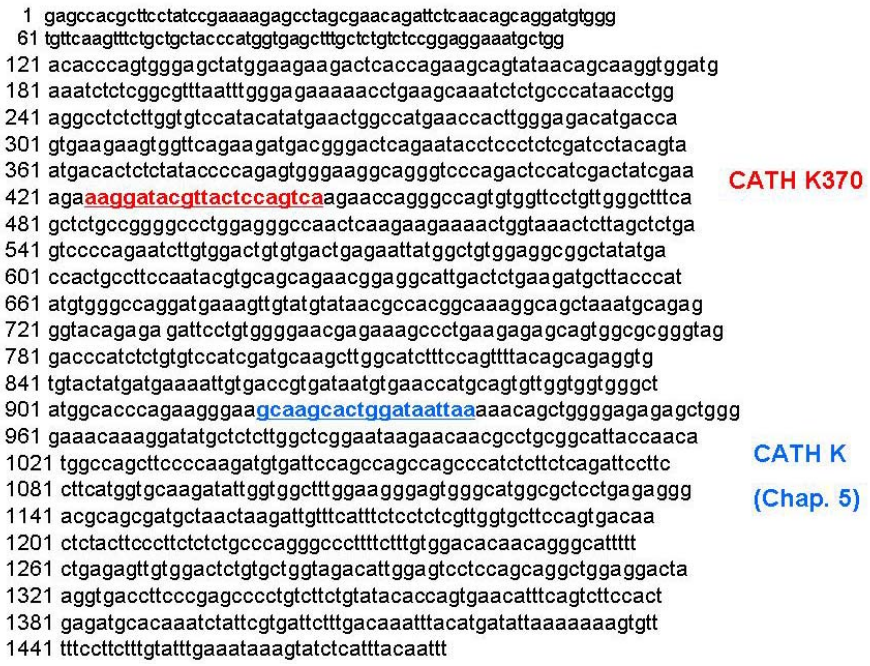
Western blots. Following shear, cells were lysed with RIPA buffer and conditioned media concentrated as above. Equal amounts of total protein were resolved by SDS-PAGE, and the blots were probed with antibodies to cathepsins L (1:500, R&D), K (1:200, Calbiochem), B (1:250, Calbiochem), or β-actin (1:1000, Santa Cruz) and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by a chemiluminescence method (11).

Results

Knocking down cathepsin K in endothelial cells affects cell health.

We used a sequence of siRNA to knockdown cathepsin K in MAEC to investigate the contribution of cathepsin K to endothelial cell elastase and gelatinase activity. MAEC were transfected with a sequence denoted as Cath K370 or a non-silencing RNA control 48 hours prior to exposure to 24 hours of St, OS, or LS conditions in serum free media. Cath K370 is different from the specific siRNA sequence used in Chapter 5 (Table 8.1).

Table 8.1 Different targets of cath K370 siRNA sequence and the other cath K siRNA sequence from chapter 5.



Pictures of the cells were captured with a digital camera after shear, and as expected MAEC exposed to LS elongated and aligned in the direction of flow. There were differences in the cells themselves, however. Those MAEC transfected with Cath K370 showed vacuole formation and membrane blebbing, particularly in the St and OS conditions while those transfected with the nonsiRNA did not show such formations suggesting that it was not due to the transfection (Figure 8.1). In another assay, we used the pharmacological inhibitor of cathepsin K (1,3-Bis(CBZ-Leu-NH)-2-propanone), and with increasing concentrations of the cathepsin K inhibitor I, there was increased endothelial cell death (Figure 8.2A). Elastase activity was inhibited by the K inhibitor, E64, and N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MK), the serine protease inhibitor (Figure 8.2B). Taken together with the siRNA results, these results

support the hypothesis that knocking down cathepsin K in endothelial cells leads to cell death.

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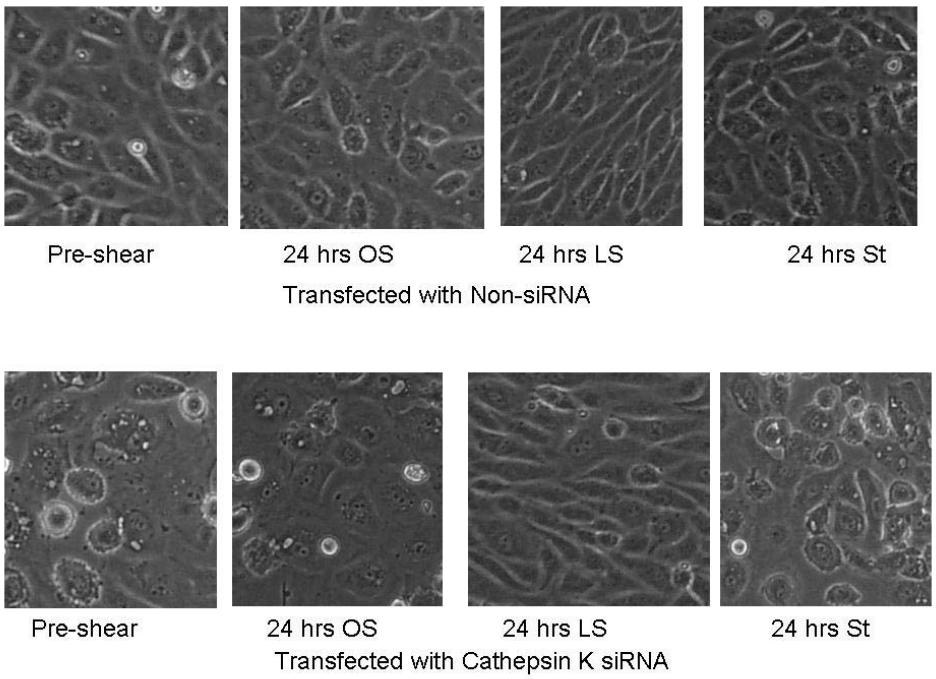

Figure 8.1. Transfection with cathepsin K 370 siRNA induces MAEC cell blebbing and vacuole formation. MAEC were transfected with Cath K370 siRNA or a non-silencing RNA control 48 hours prior to exposure to 24 hours of OS, LS, or St conditions. Representative micrographs are shown.

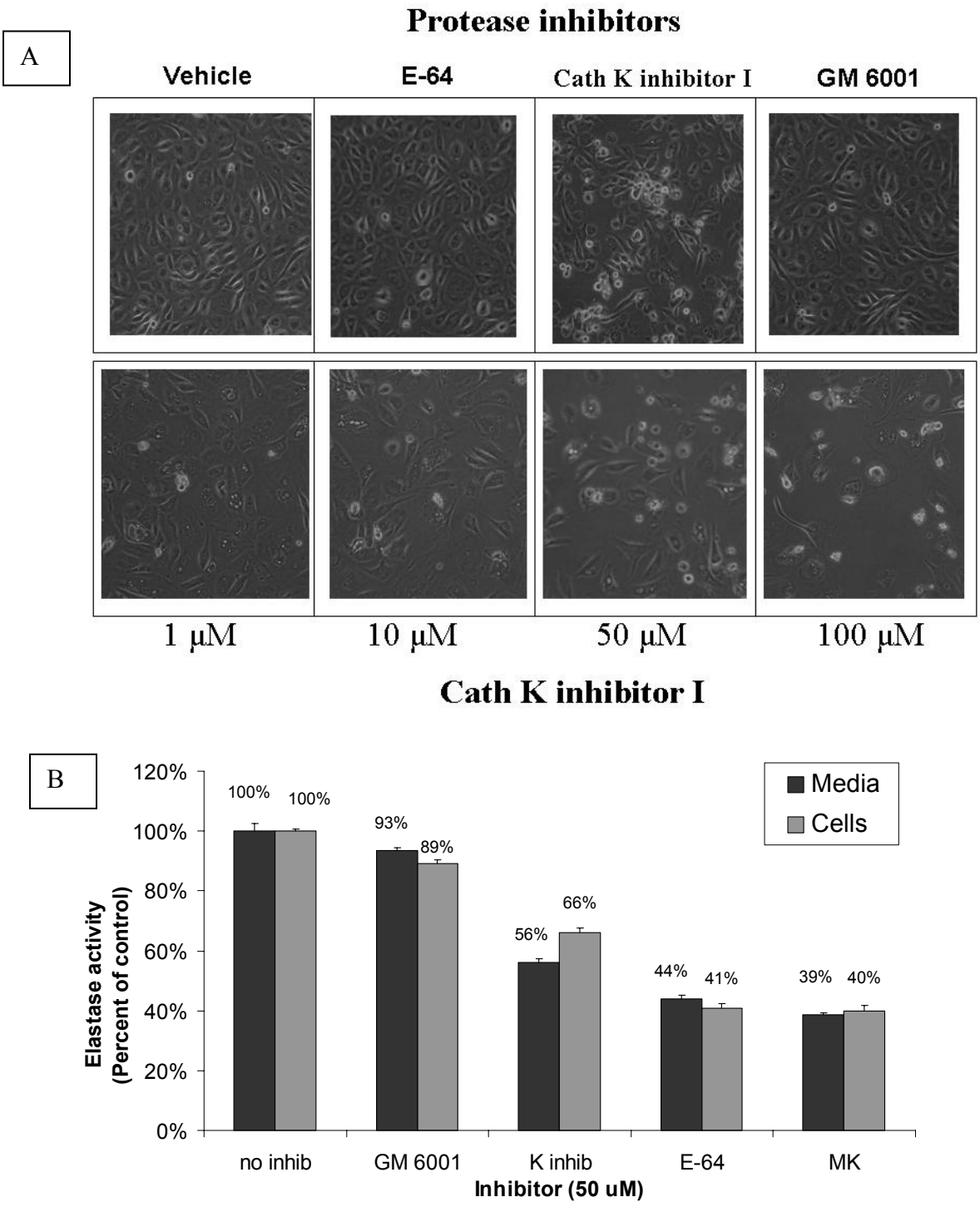

Figure 8.2. Cathepsin K inhibition with pharmacological inhibitor also induces MAEC morphology changes and cell death. Digital micrographs of cell morphology were also captured (A) after static MAEC were cultured with soluble BODIPY-elastin in the presence of various inhibitors and increasing concentrations of the cathepsin K inhibitor. Cells were also assayed for elastase activity (n=2).

Cathepsin K 370 siRNA also knocks down cathepsins B and L.

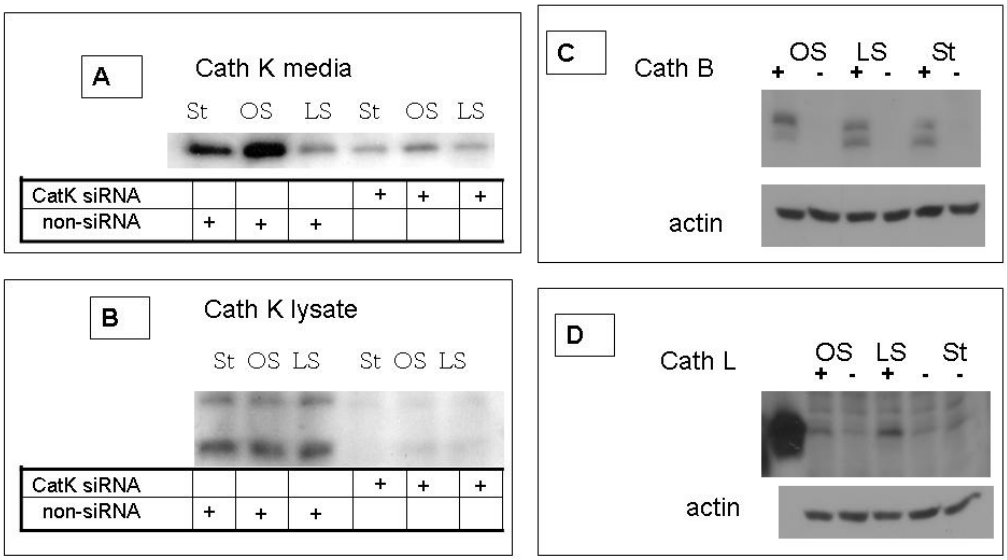

Figure 8.3. Cath K370 siRNA knocks down cathepsins B, K, and L. MAECs were transfected with Cath K370 siRNA 48 hours prior to 24 hours of OS, LS, and St conditions. Western blots were performed on the cell lysates and the conditioned media.

Since we could rule out siRNA transfection as the cause of the endothelial cell morpohological changes with the nonsiRNA result, we wanted to be sure that the loss of cathepsin K was the cause. To check the specificity of the siRNA, Western blots for cathepsins B, L, and S were performed on the cell lysates of MAEC transfected with Cath K370. Although the Cath K370 sequence BLAST did not reveal sequence homology to cathepsins B, L, or S, it was seen that the transfection cross reacted and knocked down the protein levels of cathepsins B and L as well as K under all shear conditions (Figure 8.3). Cathepsins B and L have been linked to programmed cell death in other cell types which could account for the morphological changes seen in the MAEC transfected with Cath K370. It not only knocked down protein levels of the cathepsins, but also

significantly reduced their activity in the conditioned media as seen with the cathepsin substrate Z-GPR-AMC that is susceptible to cleavage by cathepsins B and K. Conditioned media from MAEC exposed to 24 hours of OS, LS, or St conditions was assayed for Z-GPR-AMC hydrolysis. OS significantly increased cathepsin B and K activity in the conditioned media by 200% over St and LS, and knockdown with Cath K370 blunted this (Figure 8.4A).

Cathepsins K and L are known to be strong elastases, and our previous data verified their involvement in shear mediated elastase activity of MAEC, so we investigated how transfection with Cath K370, and knocking down both elastinolytic cathepsins, would affect cell mediated elastase activity. From figure 8.4B, it is clear that cathepsins K and L contribute to about 40% of the total elastase activity of MAEC. MAEC exposed to OS showed approximately two-fold higher elastase activity than that of LS, which was substantially inhibited by two different concentrations of cathepsin K inhibitor I (0.2 and 50 μM) (Figures 8.4 C and D). The lower concentration, 0.2 μM is close to the IC_{50} for this cathepsin K inhibitor with cathepsin K. It was interesting to note that the elastase activity between OS and static exposed cells were not different from each other (Figure 8.4B-D), even though the cathepsin K activity was significantly higher in OS-exposed cells than the static cells (Figure 8.4A). Western blots on the conditioned media showed that incubation with the cathepsin K inhibitor led to increased secretion of mature cathepsin K although the activity was blocked (Figure 8.5B). There was also decreased secretion of cystatin C after incubation with the cathepsin K inhibitor suggesting that there is a cathepsin K mediated function that the MAECs are trying to elicit by producing more cathepsin K and less cystatin C.

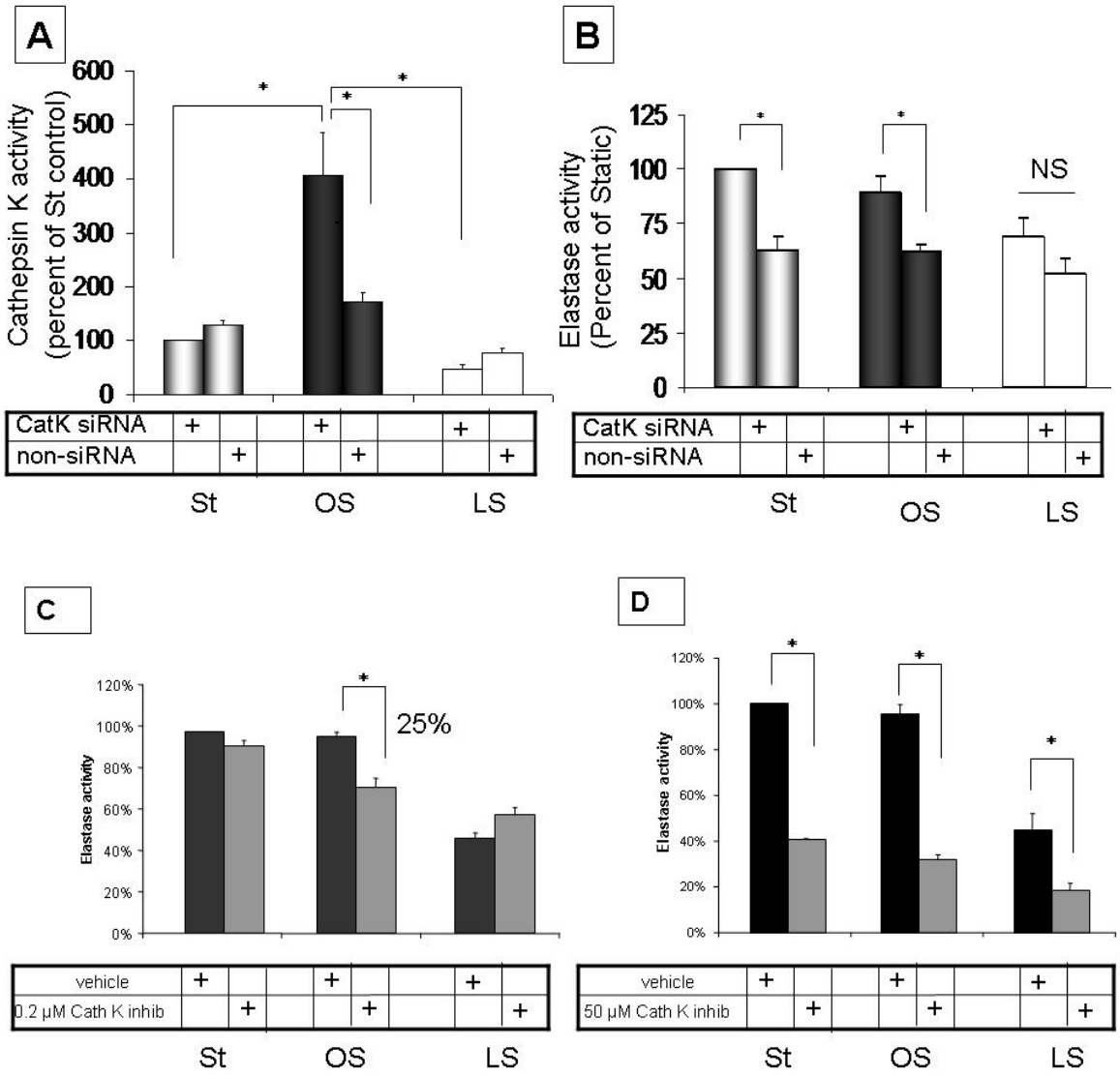

Figure 8.4. Cathepsin K 370 siRNA significantly reduces cathepsins B and K activity. After shear exposure, conditioned media was assayed for Z-GPR-AMC hydrolysis (A) and elastase activity (B). Bar graphs represent average values normalized to Static control of at least 3 independent experiments (* $p<.05$). Following 1 day exposure of MAEC to OS, LS, or static conditions, the cells were further incubated overnight under no flow conditions in fresh media containing BODIPY-elastin in the absence or presence of cathepsin K Inhibitor I at 0.2 µM (Panel C) and 50 µM (Panel D). Elastase activity, measured as an increase in fluorescence intensity, was shown as % of static control (mean ± SEM, *, $p<.05$, n=4).

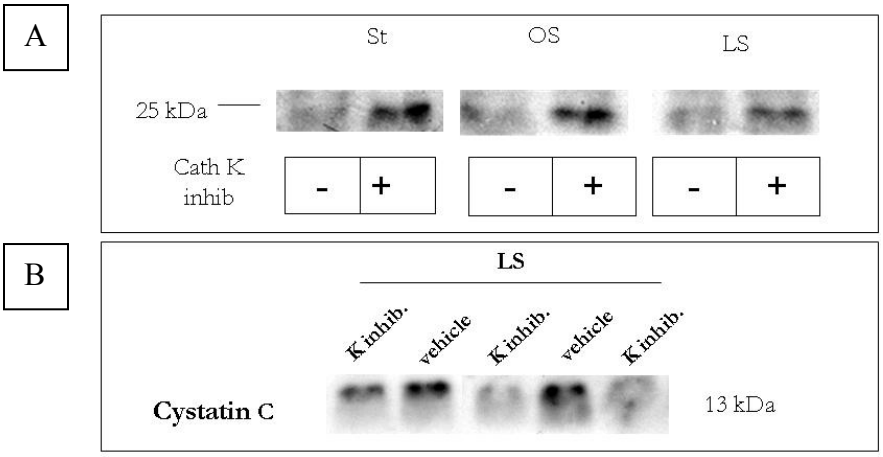

Figure 8.5. Addition of cathepsin K inhibitor increases mature cathepsin K levels extracellularly but decreases extracellular cystatin C. Western blots of conditioned media after incubation with BODIPY-elastin and 50 μM Cath K inhibitor I probing for cathepsin K (A) and cystatin C (B).

From the micrographs of the MAEC after transfection with Cath K370, it appeared that the cells were undergoing apoptosis so we employed apoptosis assays to quantify this. MAEC were transfected with either nonsilencing RNA or Cath K370 for 48 hours prior to labeling with FITC labeled Annexin V to bind phosphatidylserine that had been flipped to the outer cell membrane, a marker of apoptosis. Cells were also labeled with propidium iodide to label dead cells or cells undergoing necrosis. After FACS analysis, there was no increase in the FITC positive, propidium iodide negative gate between the nonsiRNA and Cath K370 groups (Figure 8.6).

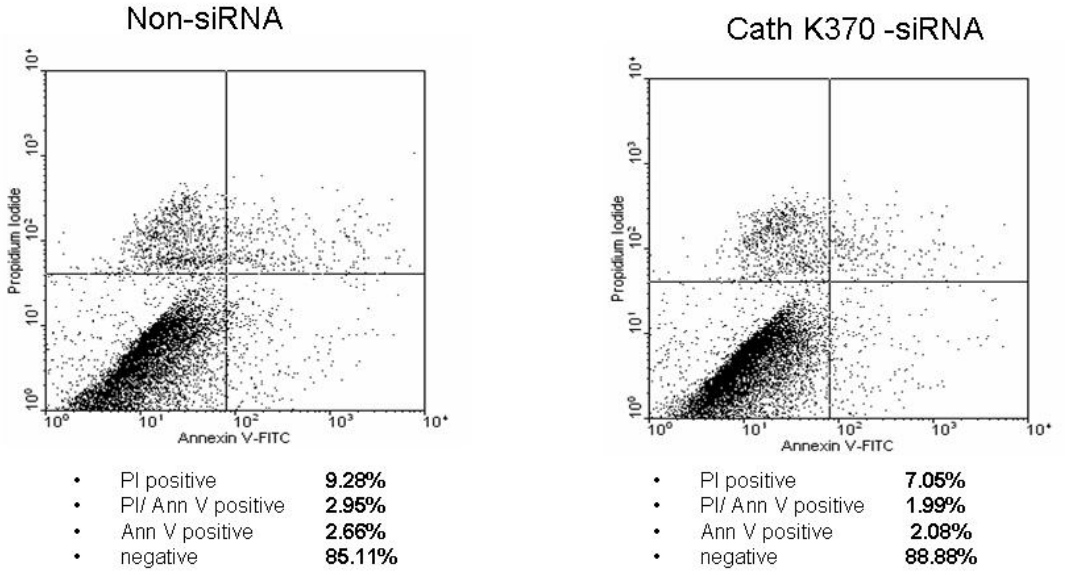

Figure 8.6. Cathepsin K siRNA induced cell death is not apoptotic.

To investigate caspase activity, we used the ApoHTS system from Cell Technology using a quenched caspase susceptible peptide. MAEC were transfected with either nonsiRNA or Cath K370 prior to shear after which, cells were scraped in PBS and lysed with the proprietary ApoHTS lysis buffer, and then incubated with the fluorescent substrate cleaved by caspases 3 and 7. From figure 8.7, nonsiRNA transfected groups had the same level of caspase 3/7 activity regardless of the types of shear stress to which they were exposed. On the other hand, transfection with Cath K370 siRNA increased caspase 3/7 activity by 50% in the St and OS cultured MAEC suggesting that since LS downregulates cathepsin activity, knocking it down further does not induce cell death (or at least does not induce caspase 3/7); cathepsin activity and expression under regions of stagnant or oscillatory flow may be necessary for cell function, of which that function is not currently known. Additionally, increasing concentrations of cathepsin K370 siRNA correlates with increased caspase 3/7 activity with R^2=0.87 (Figure 8.8).

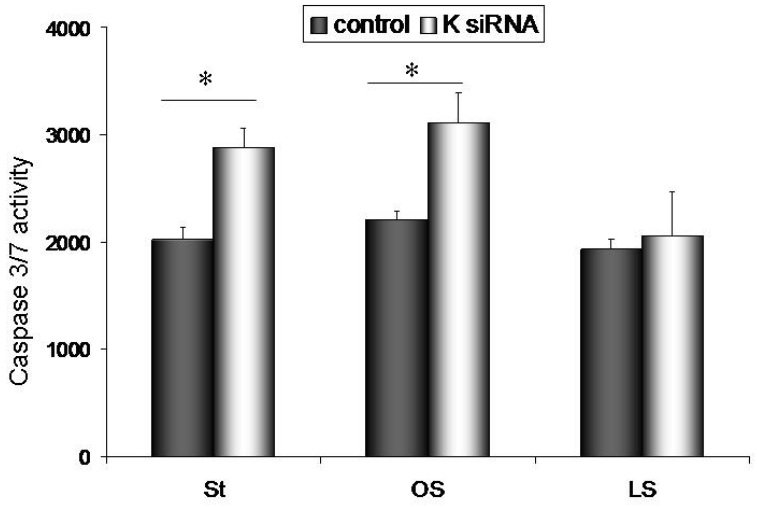

Figure 8.7. Cathepsin K 370 siRNA increases apoptosis in MAEC under static and OS, but not LS. MAEC were transfected with either Cath K370 or a nonsiRNA, sheared for 24 hours, and then lysed for use in the ApoHTS assay for caspase 3/7 activity.

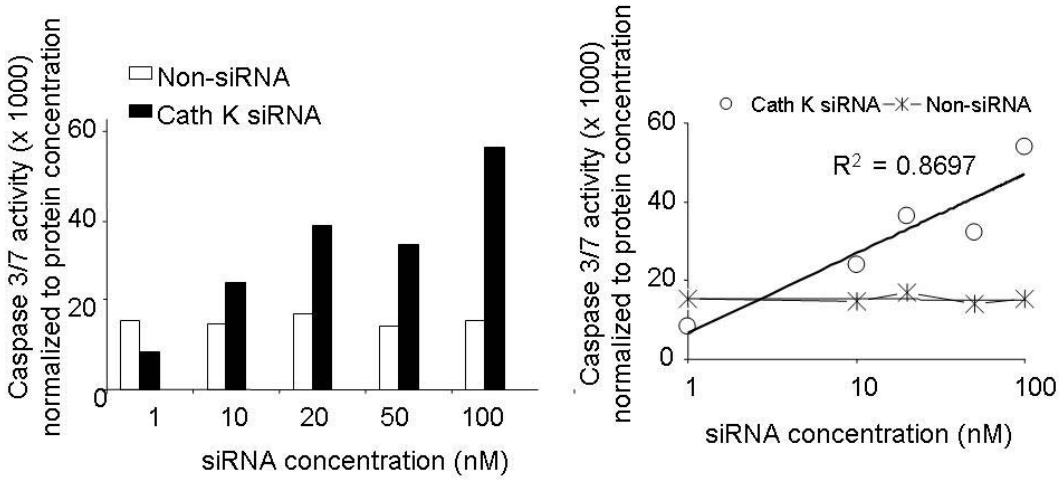

Figure 8.8. Increasing knockdown of cathepsin K induces caspase 3/7 activity.

Knocking down the combination of cathepsins B, K, and L induced programmed cell death in a caspase 3/7 independent manner.

We next tested the hypothesis that blocking caspase activity in the MAEC after transfection with Cath K370 could block the cell morphology changes indicative of programmed cell death. To do this, we transfected the cells with either the nonsiRNA or Cath K370 and then incubated them with Z-VAD-FMK, an inhibitor of caspase-1, -3, -4, and -7, Z-IETD-FMK, a caspase 8 inhibitor, or Z-LEHD-FMK, a caspase 9 inhibitor. Caspase 8 and 9 are upstream of caspase 3 and 7. Micrographs of the cells still showed the cell morphology changes in the MAEC transfected with Cath K370 indicating that programmed cell death occurs even in the presence of the caspase inhibitors. This suggests that knockdown of cathepsins B, K, and L are inducing cell death by a caspase independent pathway.

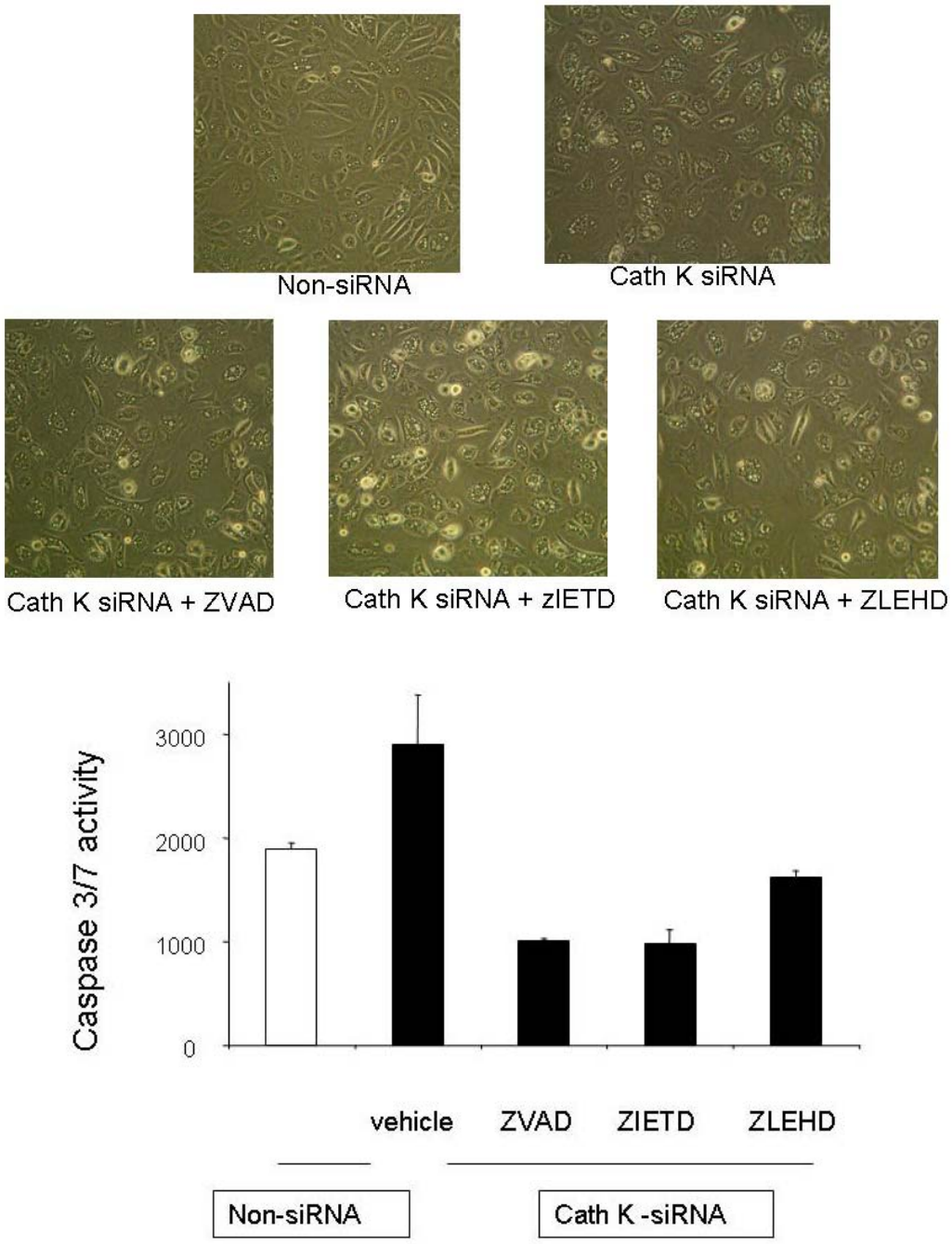

Figure 8.9. Knocking down cathepsins B, K, and L induced programmed cell death in a caspase 3/7 independent manner. MAECs transfected with Cath K370 siRNA were cultured under static conditions in the presence of the caspase inhibitors Z-VAD-FMK, a pan caspase inhibitor, Z-IETD-FMK, a caspase 8 inhibitor, or Z-LEHD-FMK, a caspase 9 inhibitor. Light micrographs (A) of the cells before lysis and Caspase 3/7 activity assay (B).

Transfected cells were stained for cathepsin K, *cis*-Golgi, and transferring receptor. Cathepsin K staining verified knockdown of cathepsin K with this sequence of siRNA as seen with the diminished fluorescent labeling when images were captured under the same confocal settings (Figure 8.10 A, B). *Cis*-Golgi was fragmented in the cells transfected with K370 compared to intact Golgi on one side of the nucleus as seen in the nonsiRNA groups (Figure 8.10 C, D). Transferrin receptors are markers for endosomes, and from the staining (Figure 8.10 E, F), the vacuolar structures induced by K370 transfection were not endosomal in nature as they do not stain positive. We further added bafilomycin A1 to block the V-ATPase proton pump that would shrink the vacuoles if they were acidic. Two concentrations were used (10 μM and 100 μM) but there was no effect on vacuole size after 1 hour (Figure 8.11 A-F).

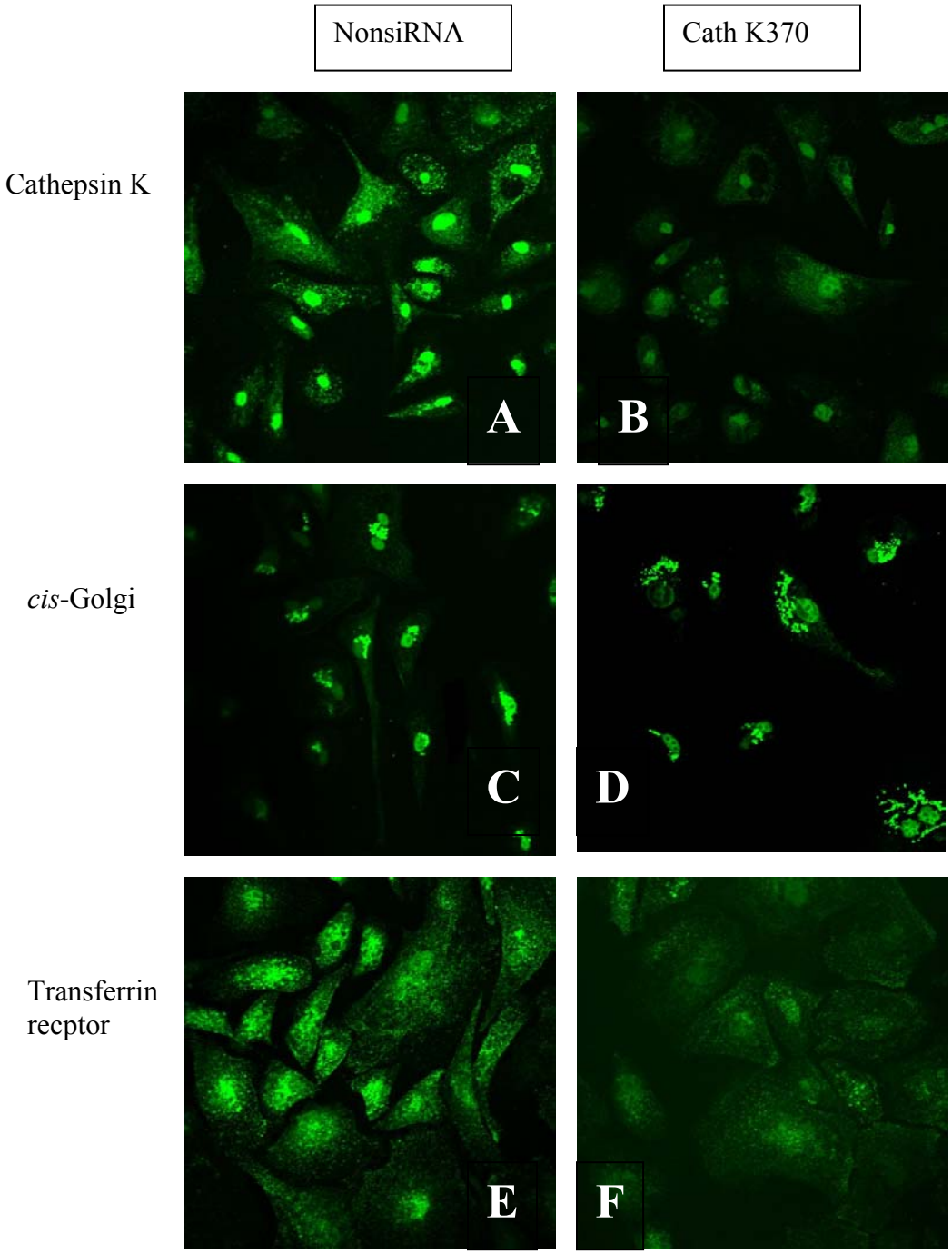

Figure 8.10. Cath K370 siRNA knocks down cathepsin K, transferrin receptor, and disrupts cis-Golgi apparatus. MAEC transfected with nonsiRNA or Cath K370 were cultured under static conditions, had their cytoplasm extracted, were fixed, and immunostained for cathepsin K (A,B), GM130 for cis-Golgi (C,D), and transferrin receptor to mark endosomes (E, F).

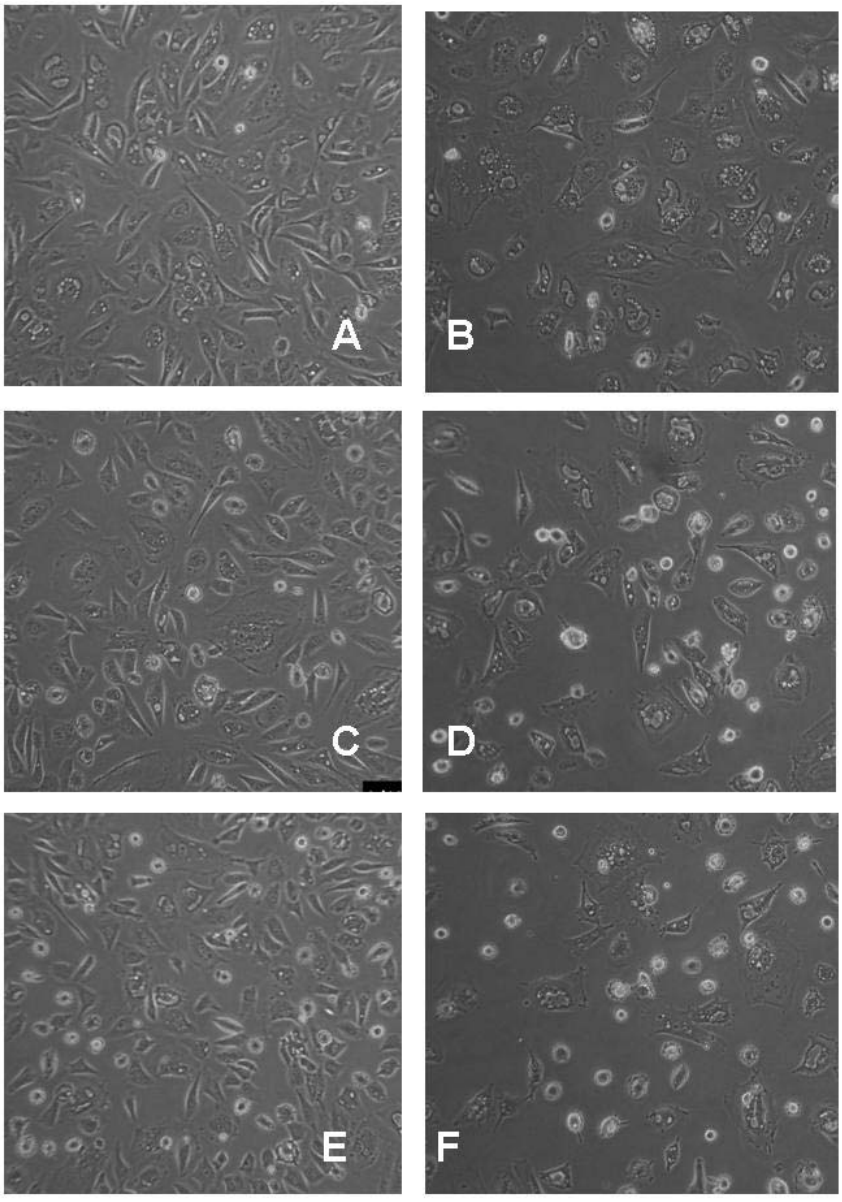

Figure 8.11. Bafilomycin A1 treatment does not shrink vacuoles induced by Cath K370. MAEC transfected with 100 nM of either nonsiRNA (A, C, E) or Cath K370 (B, D, F) were treated with 0 μM (A, B), 10 μM (C, D), or 100 μM (E, F) of bafilomycin D.

Discussion

The findings presented here show that 1) knockdown of cathepsins B, K, and L in endothelial cells with siRNA induces morphological changes indicative of a programmed cell death; 2) this programmed cell death increases caspase activity under static and OS conditions, but LS seems to protect the cells; 3) inhibition of caspases does not block the morphological changes of the MAEC; 4) a pharmacological inhibitor of cathepsin K that cross inhibits cathepsins L and S additionally induces this cell death, increases secretion of mature cathepsin K, and decreases cystatin C secretion.

OS increases apoptosis in endothelial cells (1, 12) and increases cathepsin activity (13). Several reports of cathepsin involvement in apoptosis have been reviewed (3, 4, 14) especially implicating cathepsins B, D, and L in the process. Here we see that blocking cathepsin activity either with siRNA or with pharmacological inhibition is counterintuitive by serving to increase cell death instead of preventing the completion of the apoptotic cascade. It is important to recognize that there are other forms of programmed cell death besides apoptosis that lead to similar morphology changes in cells. Caspase independent cell death and autophagy are other mechanisms by which cells commit to death in cascades that do not induce inflammation.

Autophagy is characterized by large vacuole formation, as seen in our endothelial cells after transfection with Cath K370 siRNA, caspase activation late in the process, if at all, and activation of cathepsins and other proteases (14, 15). The presence of large vacuoles does not mean that a cell is autophagic, particularly in this condition. Cathepsins are lysosomal proteases that function in the lysosome to turn over protein; cathepsin K knockout macrophages actually have enlarged lysosomes due to a loss of this

proteolytic activity (16). Other markers of autophagy such as LC3 fragmentation and Monodansylcadaverine uptake by autophagic vacuoles should be used to confirm autophagic induction in our system (15, 17). To compound the situation, it is important to note that cells undergoing autophagy do not necessarily die. It is a process used to reduce the cell volume and may be anti-apoptotic in that it sequesters small mitochondria that are usually used to trigger the intrinsic apoptotic pathway (15).

Caspase independent cell death pathways elicit some of the changes seen in apoptosis, but may not complete the entire cascade. Lockshin and Zakeri caution against assuming that the presence of caspases means that the cell death depends on caspase activity and that blocking caspases with inhibitors will not keep the cell alive (18). A cell committed to death will undergo a different pathway to get there and find a way to die. Lysosomal proteases that are leaked into the cytoplasm or the nucleus may cause damage to the cell structures without dependence on caspase activity (18) and could be responsible for numerous cell death morphologies that blur the lines between necrosis, autophagy, and apoptosis (19). Cathepsins may work upstream of the initiator and effector caspases as indicated by cathepsin activation of caspases (5), or downstream seen with caspase induced lysosomal membrane permeabilization to release cathepsins (20).

The finding here that knockdown of cathepsins B, K, and L induces a programmed cell death that is not dependent on caspase activity but does affect caspase activity based upon the shear stress condition on the mouse aortic endothelial cells, suggest the involvement of cathepsins at different stages of the cell death pathway. Laminar shear stress, known to be anti-apoptotic through nitric oxide production (21, 22) and other mechanisms protect the endothelium from traversing down a path to cell death.

Elucidating the specific death pathway in response to cathepsin knockdown could be useful in preventing endothelial cell death in atherosclerotic regions to hinder plaque rupture.

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CHAPTER 9

DISCUSSION

In total, this body of work presents data implicating cathepsins, the papain family of cysteine proteases in cardiovascular disease, and more particularly, with shear stress mediated endothelial cell responses including vascular remodeling, cell alignment, and programmed cell death. Of the numerous enzymes that comprise this family, focus has been placed on cathepsins B, K, L, and S; cathepsin K, L, and S being highly elastinolytic and collagenolytic, and cathepsin B being overly abundant in the cells. Cystatin C also serves to regulate cathepsins in the cardiovascular system as shown by its shear regulation inversely to the cathepsins suggesting a balancing act tipping the scale towards inhibition of proteases under unidirectional laminar shear stress but switching to a proteolytic environment under oscillatory shear stress by downregulating the inhibitor.

The *in vitro* endothelial work presented here offers mechanisms to the finding that atherosclerosis occurs at sites of the vascular tree that are exposed to disturbed flow conditions where the endothelium is exposed to oscillatory shear stress such as at bifurcations and sharp turns. Proteolytic remodeling of the vessel wall occurs at these sites and that is where our cathepsin balance in the endothelium switches from inhibitory to proteolytic. In contrast, straight regions of the arterial tree exposed to high unidirectional laminar shear stress are protected from plaque formation, and the endothelial cells suppress cathepsin expression and activity and highly secrete their endogenous inhibitor, cystatin C. This balancing act not only holds relevance for atherosclerosis, but also for abdominal aortic aneurysms which are also under low and oscillatory flow, and for heart valve diseases that occur on the aortic side of the leaflet

where there is turbulent and disturbed flow when the leaflets close or when stenoses and other physiological complications cause jet streams, flow eddies, and flow reversal.

To better address key targets for intervention strategies, the underlying mechanisms must be elucidated. Then, the most susceptible target or pathway can be identified to inhibit the pathological remodeling and retard plaque and aneurismal formation. Shear stress activates kinase signaling pathways in the cell that lead to gene expression. MAP kinases including ERK 1/2, JNK, and p38 lead to eNOS, Akt, and ultimately to endothelial cell functions (1, 2). Additionally, these kinases activate transcription factors NF-KB, SMAD, AP-1, c-Jun, c-fos, and c-myc to bind to their targets and turn on gene expression, of which ICAM-1 and MMP-9 are examples (3-7). Cathepsins may be the downstream result of these pathways. Already, ERK has been linked with cathepsin L overexpression in tumor cells. NIH 3T3 fibroblasts used cathepsin L for metastasis after activation of ERK and suppression of JNK; this was all downstream of ras activation and the pathway Ras to Raf to MAPKK to ERK activation (8, 9).

Promoter analysis of cathepsins K and L reveal AP-1 binding sites; (10, 11). This is a shear stress responsive transcription factor (12) that could be working to positively or negatively affect cathepsin gene transcription in endothelial cells. Cathepsin L and cathepsin B have promoters that contain Sp-1 binding sites (13, 14) which is another known shear stress responsive transcription factor (15). Methylation of the cathepsin L promoter decreases its activity (14). Studies of whether these shear responsive elements activate or inhibit cathepsin gene transcription will help explain the changes in their protein level under OS and LS.

Regulation of cathepsin activity occurs at several levels from transcription to translation, but also including cleavage of the propeptide to the mature, active cathepsin, and targeting either to the lysosome or for secretion. Once outside of the cell, the cathepsin is not in an environment optimal for its catalytic activity so outside influences must create a slightly acidic environment to turn on cathepsin function, although some studies find cathepsin activity under neutral pH (16, 17). Cathepsin secretion is not fully understood, especially since there is no signal sequence to target them for secretion, but it has been suggested that the carboxyterminus of procathepsin L has such a sequence (18). Secretory lysosomes (19) and alternative targeting pathways suggest that overexpression of cathepsins B and L overloads the lysosomal targeting (the traditional mannose-6-phosphate receptor mediated path) and causes them to be secreted instead (17, 20). Procathepsin L aggregation into multivesicular bodies occurs when it is overexpressed and may lead to mannose-6-phosphate receptor independent secretion (21). Cathepsin K is known to be secreted at the ruffled border in osteoclasts by still unidentified mechanisms (22, 23). Improved knowledge of these mechanisms may expose a target for intervention so that in cases of overexpression, such as oscillatory shear stress, the cathepsins will still not be secreted into the extracellular space where they cause disease.

Cytokines also induce overexpression and secretion of cathepsins. This has been shown to be true for macrophages, smooth muscle cells, and endothelial cells. IFN gamma increases cathepsin L mRNA in all three of the cell types while TNF alpha increases cathepsin L mRNA in SMC and EC but not in macrophages (24). Cystatin C secretion by SMCs is increased with TGF-B although there is no change in the mRNA levels (25). It also interferes with the TGFBRII to antagonize its binding of TGF-B in

tumor cells (26, 27). If the same binding occurs for SMCs, then this may be a possible negative feedback mechanism to shut down the TGF-beta mediated secretion of cystatin C.

After secretion, cathepsins must be activated. Macrophages, upon coming into contact with elastin, create tight microenvironments into which they pump protons by a vacuolar type ATPase to acidify it (28). Here, cathepsin activity is enhanced. Atherosclerotic plaque environments' pH is slightly acidic (29) and this could partially be due to macrophage infiltration and the inflammatory process. Cathepsins are capable of autocatalytically cleaving their propeptide under acidic conditions (30-32), but are also capable of cleaving the propeptide off of each other eliciting a positive feedback of more active cathepsins (30, 33) to exacerbate the proteolysis and tilt the balance. Under a disturbed flow environment with reduced cystatin C, there would be no inhibitor present to suppress their activity. Glycosaminoglycans (GAGs) complex with cathepsins to modify their activity towards different ECM substrates. In particular, chondroitin sulfate complexes with cathepsin K to increase its collagenolytic activity but suppresses its elastase activity (34, 35). This may be involved with our finding that cathepsin K siRNA shows greater reduction in gelatinase activity than elastase activity. GAG production by shear stress is not known. Endothelial cell glycocalyx GAGs in conjunction with acidic microenvironments may play a part in activation or deactivation of cathepsins in the local environment and their proteolytic potential.

Ultimately, *in vivo* animal studies to link flow with cathepsin activity must be done to validate these *in vitro* findings. Already, cathepsins K and S knockout mice crossed with the atherosclerotic model of ApoE-/- and LDLR-/- mice fed high fat diets

have produced results that implicate cathepsins in matrix remodeling and atherosclerotic plaque development (36, 37). Knockout of cystatin C in those models further supports its inhibitory role in the process as a greater number of breaks in the elastica layers and aortic dilation were seen (38, 39). The plaques in these animals occur at hemodynamically defined regions of disturbed flow, such as the aortic arch, but more definitive studies should be done to link cathepsins and disturbed flow *in vivo*. Altering flow by partially ligating the left coronary artery to cause low flow there but high flow in the right coronary artery (40) in conjunction with immunohistochemistry and cathepsin zymography can clearly test the hypothesis that high flow and unidirectional shear stress inhibit cathepsin activity and prevent pathological vascular remodeling. Arteriovenous fistula models have been done in mice and rabbits as well to generate high flows and to investigate proteolytic activity (41, 42). MMP-2 and -9 have been increased in these models compared to low flow, but this has also been verified at the endothelial cell level *in vitro* (43) where shear stress increases their activity over static conditions.

Cathepsins are "breaking out" of the lysosomes from where they were initially discovered and are not only causing disease states, but also are opening investigations into the mechanisms of different disease developments and pathological remodeling for which they were originally ignored. This work illustrates the shear stress mediated regulation of cathepsin activity in endothelial cells from mouse aortas but also has implications towards several cardiovascular pathologies which are leading killers in the United States. Selective inhibition of these powerful proteases could be key in saving lives.

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VITA

MANU OMAR PLATT

PLATT was born in Trenton, New Jersey and lived there until he was six years old. After living in Arkansas and Oklahoma, he finished his middle school and high school education in Dover, Delaware. In 2001, he graduated from Morehouse College with a B.S. in Biology and a minor in Mathematics, after which he enrolled in the Wallace H. Coulter Department of Biomedical Engineering to pursue a doctorate. When not in the lab, Platt enjoys spending time with his five brothers, of which he is the second oldest, traveling, or folding origami animals.